National Library of Canada
Acquisitions and Bibliographic Services Branch
395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada
Direction des acquisitions et des services bibliographiques
395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

In vitro PROPAGATION OF PACIFIC SILVER FIR (*Abies amabilis*) FROM EMBRYONIC EXPLANTS

by

Luiz Kulchetscki

©

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF FOREST SCIENCE

EDMONTON, ALBERTA
SPRING, 1993.

© LUIZ KULCHETSCKI 1993

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-82167-1

Canada

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF THE AUTHOR: **LUIZ KULCHETSCKI**

TITLE OF THESIS: *In Vitro* PROPAGATION OF PACIFIC SILVER FIR (*Abies amabilis*) FROM EMBRYONIC EXPLANTS.

DEGREE: **Ph.D**

YEAR THIS DEGREE GRANTED: **1993**

Permission is hereby granted to the University of Alberta Library to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves all other publication and other rights in association with the copyright in the thesis, and except as hereinbefore provided neither the thesis nor any substantial portion thereof may be printed or otherwise reproduced in any material form whatever without the author's prior written permission.

Luiz kulchetscki
Rua República Argentina,
294.
84015 - Ponta Grossa -
Paraná - Brazil.
(Phone) 011.55.422.23.3507

Edmonton, Dec. 8th, 1992

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *In vitro* PROPAGATION OF PACIFIC SILVER FIR (*Abies amabilis*) FROM EMBRYONIC EXPLANTS submitted by LUIZ KULCHETSCKI in partial fulfillment of the requirements for the degree of Doctor of Phylosophy.

Trevor A. Thorpe

T.A. Thorpe, Supervisor

F.C. Yeh, Supervisor

Victor Lieffers

V.J. Lieffers

John Beck

J.A. Beck

A. Flanagan

J. Zwiazek

David I. Dunstan

D.I. Dunstan, External Examiner
Plant Biotechnology Institute, NRC - Saskatoon

Date Nov 26 '92

ABSTRACT

A protocol is described for *in vitro* propagation of *Abies amabilis* (Dougl.). Factors such as age and stratification of the explant, concentration of BA alone and in combination with zeatin and kinetin, mineral salts, vitamins, sucrose, activated charcoal and gelling agent affected bud forming capacity and shoot elongation in cotyledonary explants. Shoot multiplication was unsuccessful and rooting was achieved in low percentage after long time under inducing treatments. This protocol has potential to produce a range of 3 to 7 shoots/cotyledon in 20 weeks. Attemps at induction of somatic embryogenesis in embryonic explants from mature seeds produced only non-embryogenic callus, characterized by clusters of highly vacuolated cells.

Histological and histochemical studies were performed on cotyledons cultivated on shoot formation (SF) and non-shoot formation (NSF) media. Cell clusters (meristemoids) consisting of 5 to 7 cells were noticeable at day 7 in the SF system. These cells developed further into meristematic domes and apical meristems. In NSF cotyledons, stomata and resin canals reached maturity, while cells within the cortex became vacuolated and developed into palisade and spongy mesophyll. Root primordia was also investigated in cotyledonary explants. A single or small group of cells located at the vascular cambium underwent periclinal mitotic divisions, and gave rise to meristemoids which continued to divide in the direction of the epidermal cells. Parenchymatic cells loaded with starch reserves, tracheid nests and vascular connections characterized the newly formed root primordia. Root mid-sections showed normal features when compared to seedling roots.

This investigation produced a protocol for labscale micropropagation of *A. amabilis,* and characterized the early events associated with dedifferentiation of cells leading to the *de novo* formation of shoots/roots. Since this species cannot be readily vegetatively propagated, this thesis makes a potentially useful initial contribution to the development of such technology.

ACKNOWLEDGEMENTS

I gratefully acknowledge financial assistance and scholarship from Brazil through CAPES - Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasília - Brasil, by which my graduate training was possible. Sincere thanks are accorded to my supervisors, Dr. Francis C. Yeh Department of Forest Science, University of Alberta and Dr. Trevor A. Thorpe Biological Sciences, University of Calgary, for their friendship, kindness and guidance and for the privilege of working with them. Many thanks to all Team Thorpe members who provided a pleasant and friendly environment during my stay at the University of Calgary.

Thanks also go to Dr. Edward Yeung for his kindness and interest in my academic preparation. I am indebted to Dr. Indra S. Harry especially for sharing her expertise and for always being there when I needed additional help and direction. I extend my thanks to the Seed Centre at Surrey, British Columbia for providing the seeds for the present study.

I thank Canada, and the many good friends that I made here, whose broad minds make this country as wonderful as it is.

This thesis is especially dedicated to my dear wife, **Sirley**, for her love, untiring support and continuous endurance through life's hardships. I am thankful for her wonderful example of perseverance and for the sweetness of her spirit.

TABLE OF CONTENT

Page

Approval page--

Abstract--

Acknowledgements--

Table of Contents---

List of Tables--

List of Figures---

List of Abreviations--

Chapter I. Introduction--- 1

Chapter II. Literature Review--- 7

Overview of Clonal Propagation--- 8

Organogenesis--- 10

Embryogenesis-- 21

Adventitious Bud Developmental Anatomy-- 23

Adventitious Root Developmental Anatomy--------------------------------------- 25

Chapter III. Materials and Methods--- 28

1. Plant Material and Culture Conditions--- 29

2. Organogenesis--- 30

2.1. Bud Induction and Elongation-- 31

2.1.1. Selection of Embryonic Explant--- 31

2.1.2. Influence of Stratification on Embryo Vigor------------------------------- 32

2.1.3. Nutrient Medium--- 32

2.1.4. Phytohormone(s) Determination-- 33

2.1.5. Time of Exposure in Cytokinins-- 34

2.1.6. Gelling Agents-- 34

2.1.7. Sucrose--- 34

2.1.8. Vitamins-- 35

2.1.9. Effect of "Pulsing" in Cytokinins--- 35

2.2. Shoot Elongation-- 35

2.2.1. Effect of Salt and Sucrose Concentrations on Shoot Elongation------------- 36

2.3. Shoot Multiplication--- 36

2.4. Adventitious Root Formation-- 37

2.4.1. Effect of "Pulsing" Hypocotyl Cuttings--------------------------------------- 38

2.4.2. Effect of "Pulsing" Adventitious Shoots--------------------------------------- 38

2.4.3. Incubation with Low Concentrations of auxins------------------------------- 38

2.4.4. Combinations of Auxin and Sucrose--- 39

3. Somatic Embryogenesis--- 39

3.1. Induction of Callus on Various Explants-- 42

3.2. "Pulse Treatment" for Callus Induction--- 42

3.3. Effect of Combinations of Phytohormones-- 43

4. Histology and Histochemistry-- 43

4.1. Plastic Embedding--- 44

4.2. Paraffin Embedding-- 45

4.3. Histological and Histochemistry Staining--------------------------------------- 45

4.3.1. Promeristemoids Formation in Cotyledonary Explants---------------------- 46

4.3.2. Apical Meristem Formation in Adventitious Shoots------------------------ 47

4.3.3. Vascular Connection Pattern in Adventitious Shoots------------------------ 47

4.3.4. Adventitious Root Primordia Formation--------------------------------------- 47

5. Data Collection and Analysis--- 48

Chapter IV. Results and Interpretations-- 49

1. Establishment of an Aseptic Culture--- 51

2. Organogenesis-- 52

2.1. Selection of the Best Explant--- 52

2.2. Explant Response to Stratification-- 55
2.3. Selection of the Optimum Nutrient Medium------------------------------------- 63
2.4. Effect of Concentrations and Time of Exposure in Cytokinins--------------- 66
2.5. Effect of Type and Concentration of Phytohormones------------------------- 66
2.5.1. Effect of Combinations of BA and Auxins------------------------------------ 68
2.5.2. Effect of Combinations of Two Cytokinins---------------------------------- 73
2.5.3. Effect of Combinations of Three Cytokinins-------------------------------- 73
2.6. Effect of Vitamins-- 76
2.7. Effect of Gelling Agents-- 83
2.8. Effect of Sucrose--- 83
2.9. Effect of "Pulsing" in Epicotyl Explants-- 87
2.10. Effect of "Pulsing" in Cotyledon and Hypocotyl Explants------------------- 89
2.11. Shoot Elongation--- 100
2.12. Shoot Multiplication--- 101
2.13. Adventitious Root Formation-- 106
3. Embryogenesis-- 116
4. Histology, Histochemistry and Light Microscopy-------------------------------- 120
4.1. Histological Analysis of Adventitious Shoot Formation *in vitro*-------------- 120
4.2. Histological Analysis of *in vitro* and *in vivo* Adventitious Roots------------- 133
4.3. Histological Analysis of *in vitro* Root-Shoot Union---------------------------- 138
Chapter V. General Discussions--- 147
1. Organogenesis-- 148
1.1. Selection of the Explant--- 149
1.2. Stratification-- 150
1.3. Nutrient Medium-- 151
1.4. Cytokinins and Time of Exposure--- 152
1.5. Vitamins-- 154

1.6. Gelling Agents-- 155

1.7. Sucrose-- 156

1.8. Liquid Pulsing-- 158

1.9. Shoot Elongation--- 159

1.10. Shoot Multiplication-- 160

1.11. Adventitious Root Formation--- 160

2. Embryogenesis--- 162

3. Histology, Histochemistry and Light Microscopy--------------------------------- 163

4. Data Interpretation-- 171

5. Summary-- 173

Chapter VI. Conclusions and Recommendations------------------------------------- 175

Literature Cited-- 181

LIST OF TABLES

Page

1. Composition for six nutrient media, as follow: Schenk and Hildebrandt (1972), as modified by Reilly and Washer (1977); Bornman (1983); Gupta and Durzan (1985); Murashige and Skoog (1962); von Arnold and Eriksson (1981); and Quoirin and LePoivre (1977)-- 40
2. Effect of age of cotyledonary explants of *A. amabilis* on their bud forming capacity and shoot elongation capacity indexes----------------------------------- 56
3. Effect of a 5x5 factorial experiment involving salt concentrations and ages of cotyledonary explants of *A. amabilis* on bud forming capacity and shoot elongation capacity indexes--- 57
4. Explant responses of *A. amabilis* to cool temperature (stratification) on their bud forming capacity and shoot elongation capacity indexes, percentual of response to phytohormones, number of shoots elongating and total number of buds per treatment--- 61
5. Effect of different times of cooling treatments on embryo vigor of *A. amabilis*--- 62
6. Effect of full- and half-strength of salt concentrations on bud induction and shoot elongation of cotyledonary explants of *A. amabilis*----------------------- 67
7. Effect of concentrations of cytokinins and time of exposure on induction and elongation of buds on cotyledonary explants of *A. amabilis*-------------------- 69
8. Effect of with and without α-Naphthaleneacetic acid incorporated at 0.1, 1.0, and 10μM and 10μM of N^6- Benzyladenine for first as the first week or second for the second week in culture for bud induction and shoot elongation on explants of *A. amabilis*-- 71
9. Effect of single or combinations of 2,4-dichlorophenoxyacetic acid at 0.1, 1.0, and 10μM and 10μM of N^6- Benzyladenine for first or second in bud

induction and shoot elongation on explants of A. *amabilis*---------------------- 72

10. Effect of kinetin at 0.1, 1.0, and 10μM applied alone or in combination with 10μM of N^6-Benzyladenine for first or second in bud induction and shoot elongation on explants of A. *amabilis*-------------------------------------- 74

11. Effect of zeatin at 0.1, 1.0, and 10μM applied alone or in combination with 10μM N^6-Benzyladenine for first or second in bud induction and shoot elongation on cotyledonary explants of A. *amabilis*------------------------------ 75

12. Effect of combinations of three cytokinins (N^6-Benzyladenine and kinetin at 10μM and zeatin at 0.1, 1.0, and 10μM) in bud induction and shoot elongation on cotyledonary explants of A. amabilis------------------------------ 77

13. Effect of combinations of three cytokinins (10μM N^6-Benzyladenine,1.0μM kinetin and zeatin at 0.1, 1.0, and 10μM) in bud induction and shoot elongation on cotyledonary explants of A. *amabilis*------------------------------ 78

14. Effect of combinations of three cytokinins (10μM N^6-Benzyladenine, 0.1μM kinetin and zeatin at 0.1, 1.0, and 10μM) in bud induction and shoot elongation on cotyledonary explants of A. *amabilis*------------------------------ 79

15. Effect of vitamin concentrations on the *de novo* organ formation process of cotyledonary explants of A. *amabilis*-- 84

16. Effect of sucrose concentrations on the *de novo* organ formation process of cotyledonary explants of A. *amabilis*-- 88

17. Effect of "pulsing" treatment with high concentration of N^6-Benzyladenine on bud induction of epicotyledonary explants of A. *amabilis*------------------- 90

18. Effect of "pulsing" treatment with high concentration of N^6-Benzyladenine on bud induction and development of cotyledons and hypocotyls explants of *A.amabilis*-- 94

19. Effect of "pulsing" treatment with high concentration of N^6-Benzyladenine and plating the cotyledons and hypocotyl explants of A. *amabilis* in solid

medium-- 95

20. Effect of a 3x3 factorial for DCR major salt concentrations (half-, a third-, and a quarter-strength) and sucrose (0, 30, and 60 mM) on elongation of adventitious shoots of *A. amabilis*-- 102

21. Effect of a 4x3 factorial for SH major salt formulation (full-, half-,third-, and a quarter-strength) and sucrose (0, 30 and 60 mM) on elongation of adventitious shoots of *A. amabilis*-- 103

22. Features of epidermal and sub-epidermal cotyledonary cells of *A. amabilis* cultured in shoot-forming and non-shoot-forming medium during the first 12 days-- 134

LIST OF FIGURES

Page

1.A. Effect of age of cotyledonary explants of *A. amabilis* on their bud forming capacity and shoot elongation capacity indexes---------------------------------- 58

1.B. General trend of bud induction and elongation on 3-day-old cotyledonary explants after 0, 1, and 6 weeks under cold treatment and high level of moisture content-- 58

2. Effect of 0 (control), 1, 2, 3, and 6 weeks of stratification period on embryo vigor and adventitious bud production of *A. amabilis*-------------------------- 64

3. Effect of combinations of three cytokinins (N^6-Benzyladenine, kinetin and zeatin) on induction and development of adventitious buds on 3-day-old cotyledonary explants of *A. amabilis*-- 80

4.A. Effect of levels of vitamins on the *de novo* organ formation in embryonic explants of *A. amabilis*--- 85

4.B. Effect of sucrose levels on the *de novo* organ formation in embryonic explants of *A. amabilis*--- 85

5. Effect of "pulsing" on axyllary bud production in different ages of epicotyl explants of *A. amabilis*--- 91

6. Effect of "pulsing" treatments on induction and development of adventitious buds in hypocotyl and cotyledonary explants of *A. amabilis*. The explants were pulse treated in high concentration of BA for 0, 1 ,2 and 3 hours and then plated on solid medium supplemented with cytokinins-------------------- 96

7. Effect of "pulsing" and plating the explants of A. amabilis on solid medium supplemented with single or combinations of phytohormones----------------- 98

8. Effect of salt formulations and sucrose concentrations on shoot elongation of *A. amabilis*--- 104

9. Prototypes of adventitious shoots of A. *amabilis* at the end of the elongation stage, displaying potential axillary shoot formation------------------------------ 107

10. Effect of different treatments to induce formation and/or development of axillary shoots in 20-week old shoots of A. *amabilis* generated *in vitro*--- 109

11. Adventitious root formation on hypocotyl explants of A. *amabilis*----------- 112

12. Expontaneous rooting and inducement of adventitious root formation on elongated shoots of A. *amabilis*-- 114

13. Callus formation on experimental approaches to generate somatic embryos on embryonic explants of A. *amabilis*--- 118

14. Light micrography of cross sections of cotyledonary explants of Pacific silver fir showing cells at the day of excision------------------------------------ 122

15. Light micrography of cotyledonary explants of A. *amabilis* showing cells at different stages of differentiation, cultivated in combination of cytokinin-containing medium--- 125

16. Longitudinal sections of cotyledons of A. *amabilis* depicting cells cultivated in shoot-formation medium--- 129

17. Longitudinal sections of cotyledons cultivated in non-shoot forming medium, showing cells at various stages of development---------------------------------- 131

18. Light micrography of transverse sections related to root formation in plantlets generated *in vitro* and normal seedlings--------------------------------- 139

19. Light micrography of transverse sections showing details of adventitious root formation in in vitro generated plantlets of A. *amabilis*------------------------- 142

20. Light micrography of transverse sections of spontaneous rooting formed in cotyledonary explants showing the anatomy of the root-shoot vascular connection--- 145

LIST OF ABBREVIATIONS

AE	von Arnold and Eriksson (1981) medium
BA	6-benzylaminopurine
BFC	Bud Forming Capacity index
°C	degrees Celsius
2,4-D	2,4-dichlorophenoxyacetic acid
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetate
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
2iP	6-(γγ-dimethylallylamino) purine
GMA	2-hydroxyethylmethacrylate
K	Kinetin; (6-Furfurylaminopurine)
L	liter
M	molar concentration
mM	millimolar concentration
ml	milliliter
µM	micromolar concentration
MCM	Bornman (1983) medium
MS	Murashige and Skoog (1962) medium
NAA	α-naphthaleneacetic acid
PEG	polyethylene glycol 400
QP	Quoirin and LePoivre (1977) medium
SEC	Shoot Elongation Capacity index
SH	Schenk and Hildebrandt (1972) medium
TBA	Tertiary butyl alcohol

v/v	volume/volume (concentration)
w/v	weight/volume (concentration)
Z	6-(4-hydroxy-3-methyl-2-butenylamino) purine or zeatin

I. INTRODUCTION

There is an unequivocal dependency of man and living organisms on forest ecosystems. This dependency clearly transposes the limits of temporal needs for any of the forest's secondary products, be they in the form of shelter, furniture, chemical products or paper. Over the past few years there has been an increasing and steady change in public concern and awareness towards our natural resources, and especially our forests. This is a natural reflection of the emphasis put in public education.

The constant search for matching both our temporal necessities, as well as the need to sustain biodiversity and consequently the environment, has created a new concept for the management of natural resources. History has demonstrated that the attempt to maintain both strategies concurrently has so far been unsuccessful. The recognition to separate these antagonist necessities has lead to two distinct types of forests: economic forests or "production forests", and "protection forests" which are set aside for environmental or conservational purposes. Therefore, economic activity should be done within the boundaries of the production forests and should be initiated at the seed production level, followed by seedling nursery establishment, site preparation and technical management, and culminated with the harvesting of the products generated in the artificial forest.

Using this approach, silviculture, biotechnology, tree improvement and forest management are the technical tools for creating and directing this artificial forest towards its ultimate goal. This forest should have all the necessary components for its survival and high performance within a long term plan, as it is characteristic of any forestry program. Desirable characteristics for the new forest would include superior wood or fiber quality, resistance to disease, resistance to soil salinity and/or high soil acidity and low nutrient content, frost resistance, rapid growth and high wood density (Zobel and Talbert, 1984; Schuch, 1991). Techniques such as genetic screening and gene manipulation present potential in plant biotechnology and will have a measurable impact in achieving these goals (Schuch, 1991). Some of these important traits are found naturally in the wild within isolated individuals; in such cases, mass propagation of these selected individuals through tissue culture represents a potential tool for the complete capture of the additive and non-additive traits.

However, particularly for conifers, this promising approach has been impeded since mature tissues are less responsive to the *"de novo"* morphogenetic process. In addition, the high cost of micropropagated plantlets, in comparison to seedlings, creates limitations for the widespread use of micropropagation (Thorpe and Harry, 1990). A variety of methods have been tried to deal with the problem of non-response in mature explants. These include hedging, the use of stump sprouts, the practice of coppicing (in hardwoods), and rejuvenating treatments (Thorpe and Harry, 1990). Due to the limited success obtained in promoting rejuvenation, a partial solution to this problem is the use of juvenile explants, such as immature and mature embryonic explants.

Seedlings are the primary planting stock for the forest industry and the most reliable tool for mass production of improved seeds. The incorporation of single or multiple genetic traits is through the pursuance of long and continuous tree improvement programs. However, genetically improved seeds of conifer species are not readily available, as most existing seed orchards are in the first or early second generation (Thorpe, 1983). Also, the establishment, maintenance and management of productive seed orchards is very costly and demanding, and subject to fluctuations in seed production due to a variety of factors, especially unpredictable climatic changes.

There is a reasonable expectancy that tree improvement, in the future, will involve molecular biology. If successful, the insertion and deletion of multiple-genes will be the preferred way for planting stock production in reforestation programs. Since woody traits are normally encoded by multiple genes, their characterization, isolation and insertion into the genome still represent a challenge not yet achieved. Concomitantly with this problem, gene transfer to plants other than those considered easy experimental models (such as *Nicotiana tabacum*, *Petunia hybrida* and *Arabidopsis thaliana*) are not easily mediated through *Agrobacterium tumefaciens* or *A. rhizogenes* (Potrykus, 1991). Here again, micropropagation will be necessary for the mass clonal propagation of the improved transgenics. However, while modern tools such as tissue culture, molecular biology and somatic cell genetics are the subject of much interest and attention, for the future economic forest the natural forest has to be maintained as a permanent reservoir of DNA and biodiversity.

Vegetative propagation of conifers for reforestation now accounts for an annual production of at least 65,000,000 plants worldwide (Ritchie, 1992; Talbert *et al.*, 1992); this represents an area of approximately 39,000 hectares of commercial reforestation using a theoretical spacing of 2.0 m x 3.0 m. Such programs make economic sense when supplies of high value genotypes or seed are limited. In such situations a bulking program using rooted cuttings can make it economically feasible to use in reforestation (Ritchie *et al.*, 1992). In Canada, forestry directly and indirectly employs 10% of the labor force, and injects some $ 36 billion annually into the economy (Dunstan, 1988). Thus biotechnological approaches for forest renewal are of such importance for the country.

Clonal mass propagation *in vitro* of the most economically important conifers is still difficult, time consuming and an expensive work. However, direct economic application looks promising, since the genetic gain from elite clones at the final rotation has indicated good possibilities of paying-off the investment in forestry. An analysis based on the assumptions of sensitivity to the net present value to cost, gain and available technology (Timmis, 1985) shows that the final product, which originated through clonal propagation, is more sensitive to genetic gain than to cost. This means that vegetative propagation and more advanced biotechnologies applied at several times the current cost could still pay off. Probably, a significant portion of these dividends will come from shortening the long rotation period or breeding cycles, as technical advances permit. It is also expected that since clonal forestry has the potential to optimize yield and shorten rotation time, the area set aside for reforestation should be smaller if the current demand is maintained. This is particularly important when a priority is laid on agricultural production to feed a hungry and ever increasing world population.

Abies amabilis (Dougl.) Forbes belongs to the Pinaceae family, and is commonly referred to as Pacific silver fir, amabilis fir, cascade fir, white fir and silver fir. It occurs in Southeastern Alaska, coastal region of British Columbia and the coast and cascade ranges of Oregon and Washington (Franklin, 1974). *A. amabilis* is one of nine North American species used as ornamentals, christmas trees, lumber, and in some cases as plywood. It is also a valued source of pulpwood, and so is being currently used in commercial reforestation programs. Pacific silver fir forests also have ecological and scenic value, providing cover for mountain watershed and occupying sites which are critical for the maintenance of high-quality, well-regulated streams (Franklin, 1974).

Although commercial propagation is exclusively by seed, there are a number of problems associated with sexual propagation, two of which are genetic variability among seedlings and a limited seed supply. For *A. amabilis*, seed production is low and seasonal, and requires a long time and much work to collect, (mainly due to the fact that native stands serve as the primary seed source). Also seed orchards are not yet available. Thus a protocol for microprogating this species would be advantageous as an alternative way for mass propagation of trees with superior morphological and physiological characteristics and would eliminate many of the difficulties associated with sexual propagation.

The objective of this present study was to develop a protocol for *in vitro* propagation (organogenesis and embryogenesis) of *A. amabilis*. Any information obtained could serve as groundwork for research into operational vegetative propagation utilizing somatic tissues from trees in adult phase, and for understanding the possibilities and constraints of applying this protocol into commercial reforestation and the establishment of seed orchards. Besides its potential for mass propagation of genetically improved seeds, somatic embryogenesis in particular could also be of benefit as a potential source of inoculum for further genetic transformation. To carry out this objective, research was conducted using embryogenic tissues. Effects of explant type, physiological age of the explant source, phytohormones and their concentrations on adventitious bud formation and somatic embryogenesis were investigated. In addition, subsequent rooting of shoots was investigated.

A second objective of this study was the application of histological techniques to determine the normalcy and viability of the plantlets produced *in vitro,* as well as to get a better understanding of the *de novo* morphological process(es). Particular emphasis was placed on establishing a reliable timetable of the histological events, starting with the first cell division, promeristemoid formation and development, and culminating with adventitious shoot and root formation.

II. LITERATURE REVIEW

Overview of Clonal Propagation

Vegetative propagation is a long-standing horticultural and forestry practice. This technique is based on the concept of totipotency, by which competent cells undergo dedifferentiation when subjected to external stimuli (physical environment and nutrient medium). Such cells demonstrate their intrinsic capacity for organized development, which is ultimately the reflection of selective gene activity (Thorpe, 1983). *In vitro* cloning offers some advantages such as unlimited mass production of uniform and genetically identical individuals and reduced risk of introduction or loss of some features or traits, which is a common problem in plant breeding (Thorpe *et al.*, 1991). The expectation that *de novo* organ formation can be induced in a reproducible manner and without variation has been the goal of many researchers attempting to mass propagate selected plants. This approach is especially attractive for woody plants which tend to be outbreeders (Thorpe and Hasnain, 1988; Thorpe *et al.*, 1991). The most frequent methods employed for vegetative propagation of forest trees are the use of rooted cuttings, rooted needle fascicles, also known as brachyblasts, and grafting (Thorpe and Biondi, 1984; Harry *et al.*, 1987; Thorpe and Harry, 1990; Thorpe *et al.*, 1991). These very attractive, promising and sometimes even simple techniques are frequently characterized by a rapid loss of rooting capacity with increasing maturity of the explant. Also, factors such as speed of response to rooting factors, number and length of adventitious roots and overall survival rate and growth decline with increasing age of the explant (Thorpe, 1983; Thorpe *et al.*, 1991). The fact that *in vitro* clonal propagation presents the most advanced application of plant tissue culture (Murashige, 1978; Thorpe, 1983) together with the recognition that clonal planting stock offers many benefits, since one can obtain true-to-type individuals from their mother explant, has led to much recent research in micropropagation of woody angiosperms and gymnosperms (Thorpe and Biondi, 1984; Thorpe *et al.*, 1991).

Morphogenesis can be promoted through embryogenesis or organogenesis (Dunstan and Thorpe, 1986). The former involves formation of bipolar structures (shoot/root axis) and presents the best potential for producing the largest number of plantlets. In contrast to embryogenesis, organogenesis leads to *de novo* formation of meristematic *loci* with organization of well defined shoot and/or root primordia (Vasil and Vasil, 1980). In this case, the unipolar bud primordia can be induced on the explant directly or through an intermediate callus stage (Dunstan and Thorpe, 1986). The shoot or root primordium is said to arise from one or a small group of cells (Torrey, 1966; Smith and Thorpe, 1975; Vasil and Vasil, 1980; Villalobos *et al.*, 1985). Although the potential for mass propagation with asexual embryogenesis is greater, the more laborious and time-consuming multi-stage organogenetic pathway is most widely used (Thorpe and Biondi, 1984). The reason for this apparent incongruency is that somatic embryogenesis is achieved less frequently than organogenesis, particularly for conifer species (Thorpe and Hasnain, 1988; Tautorus *et al.*, 1991).

Mass propagation through organogenesis can be divided into four stages: (I) initiation of shoot buds, (II) development of buds into shoots, (III) rooting of shoots, and (IV) preparation of plantlets for planting out (Dunstan and Thorpe, 1986; Harry *et al.*, 1987; Pulido *et al.*, 1990; Thorpe *et al.*, 1991). Each one of these stages is achieved through the careful selection of the inoculum and empirical manipulation of the physical environment and the nutrient medium (Thorpe, 1983; Dunstan and Thorpe, 1986). Extensive studies conducted on a large number of species support the idea that the *de novo* organ formation from primordia into complete organs and plants resembles basically the same natural process found in intact plants (Thorpe, 1983).

After the careful selection of each critical component to promote the *de novo* morphogenetic process in competent cells, the responsive explants will be redirected to two possible general pathways: activation of a possible single cell to divide periclinally towards meristemoid formation, followed by a meristematic zone, bud primordia, shoot development and finally the formation of plantlets, or through the *de novo* formation of polarized structures, named somatic embryos (Thorpe, 1983; Thorpe *et al.*, 1990; Dunstan, 1988).

So far, the various studies performed *in vitro* employing a variety of explants in numerous plant species have shown that successful culture and organized development require that the appropriate nutrient medium and culture conditions be provided to the selected sterilized explant (Murashige, 1978). Basically, five classes of compounds in the nutrient medium play an important role for induction of the *de novo* organ formation in most plant species, namely: (1) phytohormones; (2) inorganic macro- and micro-nutrients; (3) carbon and energy source; (4) vitamins; and (5) reduced nitrogen. These components in combination with physical factors such as the liquid or semi-solid nutrient medium, pH, light and temperature play a critical role in controlling dedifferentiation and subsequent *de novo* organized development (Thorpe, 1983).

The basic principles of the *de novo* organ formation *in vitro* were established by Skoog and Miller (1957). They stated that organ differentiation in plants is mainly regulated by an interplay of two groups of hormones, namely auxins and cytokinins. These phytohormones usually act sygnergistically such that a high auxin to cytokinin ratio favors root formation, and a low auxin to cytokinin ratio brings about the development of shoot buds (Skoog and Miller, 1957). All components known to play a role in the *de novo* organ formation *in vitro* such as physical factors, nutrient medium and explants must be determined experimentally, since it is known that each inoculum has different requirements (Dunstan and Thorpe, 1986).

Organogenesis

Almost a quarter of century ago, Hartmann and Kester (1968) ranked plant species in groups ranging from those easily propagated by cuttings to those propagated with difficulty. In their long list they placed *Pinus* and *Abies* as the most difficult species to propagate. Later, Bonga (1977), reviewing the same topic, agreed with the previous list. It is also significant that twenty two years later, in the most recent review list of micropropagated plants (angiosperms and gymnosperms) through organogenesis (Thorpe *et al.*, 1991) only *Abies alba*, *A. balsamea* and *A. fraseri* have been listed as the successfully micropropagated firs *in vitro*, although they showed severe limitation in various aspects when their organogenetic protocol was compared with other coniferous species. Today, despite successful propagation of many coniferous species by tissue culture, there are few reports involving species of *Abies* on organogenesis (Bonga, 1977; Saravitz *et al.*, 1987; Zygmont and Schwarz, 1987) and embryogenesis (Schuller *et al.*, 1989; Tautorus *et al.*, 1991). Furthermore, the potential number of plantlets formed per explant is among the lowest recorded for conifers.

One of the earliest successes of *in vitro* micropropagation of conifers was accomplished by Cheng (1975) using embryonic explants of Douglas fir subjected to an elevated concentration of exogenous cytokinin (0.5 to 1.0 mg/l), before subculturing them onto a nutrient media devoid of phytohormone. Following her achievement and that of Sommer *et al.* (1975) with *Pinus palustris*, the literature has seen numerous protocols for mass propagation of conifers through the organogenetic pathway. Later (Reilly and Washer,1977) described for the first time the differentiation of plants from cotyledonary and hypocotyl tissue of fully mature or developed embryos of radiata pine (*Pinus radiata*). This protocol was attained employing Schenk and Hildebrandt (1972) nutrient medium with a few modifications plus cytokinin as the inductive factor. Approximately the same time, Cheng, (1977) provided details by which adventitious buds were generated from practically every organ and tissue of Douglas fir, such as excised embryos, hypocotyls, cotyledons, needles, and stems. However, cotyledons were chosen as a model system for investigating various factors, including the morphogenetic process of the *de novo* organ formation. In conifers, juvenile explants obtained from embryonic explants are normally more responsive for the inductive treatments (Thorpe and Hasnain, 1988). In investigating the possibility of increasing the potential of mass propagation in radiata pine (Aitken *et al.*, 1981) separated three main inocula: 1) whole excised embryos, 2) excised cotyledons from embryos after a period of elongation in culture and, 3) excised cotyledons from aseptically germinated seeds. They came to the conclusion that cotyledons from germinated seeds were a much better source of inoculum than the other remaining explants. Biondi (1980) considered that cotyledonary explants presented basically two advantages: 1) homogeneous initial explants, consisting of one type of cell, except for vascular initials and 2) free from a callus formation phase. Studies with embryonic explants in *Pinus canariensis* have shown that cotyledons showed the best potential for *in vitro* mass propagation. This is due to the possibility of better control of asynchrony in adventitious budding, as well as the fact that cotyledons have a large surface per volume and are also to handle (Pulido *et al.*, 1990). Also, results employing this same type of inoculum for radiata pine have revealed that after three weeks in culture, meristematic tissue was visible on the cotyledonary surfaces in contact with the medium (Aitken *et al.*,1981). They further observed that meristematic cells had proliferated from the epidermal and hypodermal layers of cells (Yeung *et al.*, 1981).

Although cotyledons have been the most selected and responsive juvenile explants for mass propagation of conifers (Mott *et al.*, 1977; Patel and Thorpe, 1984) there are some exceptions for this general rule. In the process of mass propagation of firs from cotyledonary explants, some representatives of this species have shown a lack of response to cytokinins. Saravitz *et al.* (1991) reported that, unlike the general trend for coniferous species, cotyledons of Fraser fir rarely produced adventitious buds *in vitro* and bud formation occurred mainly in hypocotyls. Zygmont and Schwarz (1987) also noted formation of buds on hypocotyls and not on cotyledons. Despite bud induction on hypocotyls of Fraser fir, the actual number of buds per explant is much less than that reported for most conifers (Saravitz *et al.*, 1991).

The chemical composition and physical make-up of the nutrient medium are the determining factors in the initiation and development of shoots *in vitro*. Although there is generally no single medium that must be used for a given species or type of culture, any application of *in vitro* technology to the study of organogenesis usually involves medium optimization as one of the first steps (Brown and Thorpe, 1986). A significant influence of the nutrient medium on morphogenesis in various conifers has been documented, where the frequency and yield of bud induction have been affected by the major salt formulation employed in the nutrient medium (von Arnold and Eriksson, 1981; Berlyn and Beck, 1980; Patel and Thorpe, 1984; Patel *et al.*, 1986; Patel and Thorpe, 1986, Harry *et al.*, 1987; Pulido *et al.*, 1990). An examination of the composition of media generally used for the culture of plant cells reveals that they contain NO_3^- and NH_4^+, which are the sources of nitrogen for growth (Dougall, 1972). An examination of media for the growth of cells from particular species or specific cell lines shows that there are quite large differences in the ability to grow with these two ions as nitrogen sources. Different medium formulations normally vary either in the type or concentration of the nitrogen source, other macro or micro elements, or the organic components (Gamborg *et al.*, 1976). Results obtained with embryonic explants of *Pinus elliottii* have suggested that *de novo* organ formation was limited by the presence of ammonium and high nitrogen concentrations (Pérez-Bermudez and Sommer, 1987), and others have also reported best results when ammonium was ommited or drastically reduced in the media (Sommer *et al.*, 1975; Sommer and Caldas, 1981; Konar and Singh, 1980). Flinn *et al.* (1988) working with *P. strobus* reported that high ratios of NO_3:NH_4 favored callogenesis, while those with low ratios avoided callus formation. Phosphate plays an important role in the medium since it serves as a buffer for controlling the pH of the medium. During the growth of cultured plant cells, the pH of the medium changes; thus, it may drop as low as pH 4 and rise to pH 7. Such changes can be expected to have an impact on the metabolism of the cells (Dougall, 1972).

Often dilutions of the standard media in proportional strength are used for certain stages such as organ induction and organ development (Bornman, 1983). Varying the strength of the basal medium affected the frequency of bud induction in *P. contorta*, with a quarter-strength giving the optimum response (von Arnold and Eriksson, 1981). Full strength of MCM medium (Bornman, 1983) was the best for induction of buds on *P. canariensis*; however, half strength was optimum for bud development (Pulido *et al.*,1990). The best bud induction medium for *A. fraseri* was Brown and Lawrence (BLG) medium, as modified by Amerson *et al.* (1985), as reported by Saravitz *et al.* (1991).

Mott *et al.* (1977) observed that as seed germination of loblolly pine proceeded, adventitious bud regeneration on cotyledons reached a maximum and then decreased until the potential for regeneration was lost. This reduction of bud forming capacity with increasing age has been reported in many other different coniferous species for different kind of embryonic explants (Reilly and Washer, 1977; Aitken *et al.*, 1981; Jansson and Bornman, 1981; Biondi and Thorpe, 1982; Abdullah *et al.* 1987; Toivonen and Kartha, 1988; Pulido *et al.*, 1991; Saravitz and Amerson, 1991).

The chronology of the morphological changes and patterns of buds initiated at ultrastructural levels on cotyledonary explants of *P. radiata* (Reilly and Washer, 1977; Yeung *et al.*, 1981; Villalobos, 1983) showed that responses to *de novo* organ formation have close association with the cotyledons' anatomy. Several significant physiological and morphological changes happen in cotyledonary explants with increasing age, such as development of stomatal complexes, increasing maturation of the resin canals, specialization of the subepidermis cell layers into mesophyll and palisade parenchyma, alteration of protein content, and thickness of the cell wall (Bornman, 1983; Villalobos, 1983; Aitken-Christie *et al.*, 1985; Patel and Thorpe, 1986; Pulido *et al.*, 1990). The progressive specialization of the cells and tissues probably exert a strong limitation on the plasticity and the capability of the totipotent targets cells to respond to the dedifferentiation signals (Thorpe and Biondi, 1981; Aitken-Christie *et al.*, 1985; Abdullah *et al.*,1987).

Cooling or stratification treatments are rarely reported in *in vitro* protocols but have been demonstrated to improve seed germination *in vivo* substantially, since dormant or immature seeds germinated slowly and unpredictably (Leadem, 1986). Harry *et al.*, (1987) reported that stratification of seeds of eastern white cedar for up to 30 days before *in vitro* culture did not improve subsequent propagation. For fully mature, well-developed conifer seeds, moist storage at 2°C for three or more weeks (stratification) is the most effective means of breaking dormancy and enhancing germination speed and uniformity. "Naked" stratification, i.e., chilling imbibed seeds in bags without soil or other media (Allen and Bientjes, 1954), is the method prescribed for most tree seeds by the Association of Official Seed Analysts (Anonymous, 1978) and the International Seed Testing Association (Anonymous, 1985). Recently, stratification-redry, a modified method in which seed moisture level is controlled during the chilling period, has been found more effective for some tree seeds. Although initially proposed as a means for storing stratified *P. ponderosa* and *Pseudotsuga menziesii* seeds (Danielson and Tanaka, 1978), stratification-redry was soon found to improve the germination of *A. grandis, A. amabilis,* and *A. lasiocarpa* seeds that did not respond very well to usual stratification methods (Edwards, 1980). The advantage of the stratification-redry technique (Edwards, 1981) is that moisture levels are low enough to prevent radicle protrusion during prolonged chilling, but remain sufficiently high to maintain the metabolic and cytological processes normally occurring during stratification (Côme and Thévenot, 1982). This technique has been tested on *A. amabilis* seeds in the laboratory and in the nursery. In the laboratory, the stratification-redry technique, relative to 4 weeks stratification, increased germination speed and total germination in 30 different lots (Leadem, 1986). Nursery tests conducted on *A. procera* showed little difference between the redry technique and 2 months stratification when seeds were sown under warm conditions, but during cold, wet conditions, seeds that received the redry treatment germinated significantly better. Leadem (1986) observed that different results were obtained under warm and cold growing conditions and suggested that temperature should be examined as a factor in the germination response of *Abies* seeds following stratification.

Seed source has also been found to influence the efficacy of chilling treatment in seeds of various conifers (Heit, 1961; Hellum, 1968; Wang, 1978; Edwards, 1980; Sorenson, 1980). The relationship between stratification and lot vigour (one probable cause of such seed source variation), together with chilling method, seed source, collection year, germination temperature, and seed vigour all affected the germination response of *A. amabilis* to stratification (Leadem, 1986).

The observation made by Cheng (1975) that BA is a requirement for shoot formation in Douglas fir was supported by Winton and Verhagen (1977) and today it represents the most common cytokinin employed for the *de novo* organ formation process in gymnosperms. The term cytokinin is used to encompass compounds which promote cell division in callus cultures of plant tissues. The archetype is N^6-furfuryl adenine, known by the trivial name, kinetin. The naturally occurring cytokinins are N^6-substituted adenine derivatives, known by the acronym, 2iP and the trivial name, zeatin. These natural compounds together with BA and kinetin are the cytokinins commonly employed in plant tissue culture (Dougall, 1976). Generally, cytokinins are essential for induction and maintenance of organized development that leads to adventitious bud formation (Thorpe and Biondi, 1981). The type of cytokinin used to induce bud formation is important, and conifers may differ in their *in vitro* requirement (von Arnold, 1982; Rumary and Thorpe, 1984; Abdullah *et al.*, 1987). In *P. radiata* cotyledons, cytokinin and light factors were required in order to promote the developmental behaviour of the excised cotyledons (Villalobos *et al.*,1984a). Macromolecule synthesis was more visible at the epidermis and first and second rows of mesophyllic cells as opposed to cotyledons cultured in the absence of cytokinins (Villalobos *et al.*,1984b). A specific concentration of cytokinins or lack of them will promote the optimal conditions for all the multi-stages of each selected explant. In pitch pine embryonic explants, inclusion of 5μM kinetin in the medium was found to be optimal. Higher concentrations caused decline in the frequency of bud formation and poor growth (Patel *et al.*, 1986). In *P. canariensis* cotyledonary explants, the inclusion of 10μM BA gave optimum shoot formation in comparison with kinetin, 2iP or zeatin at similar concentrations (Pulido *et al.*, 1990).

Superior effects of mixed cytokinins were reported in various conifer species, like *Pseudotsuga menziesii* (Cheng, 1975), *Picea mariana* and *P. glauca* (Rumary and Thorpe, 1984), and *Thuja occidentalis* (Harry *et al.*, 1987). In *Pinus strobus*, Flinn *et al.* (1988) observed that BA and 2iP induced a large amount of caulogenesis, with BA being more potent than 2iP. Pulido *et al.*, (1990) observed that when BA was employed in combination with 2iP, kinetin or zeatin at 10μM, the percentage of cotyledons forming buds and the average number of buds per cotyledon were higher than application of a single cytokinin. In epicotyl explants of *P. glauca* and *P. mariana*, when a mixture of BA and 2iP in equimolar concentrations of 5μM was used, a synergistic interaction between the two cytokinins were observed as compared to BA alone (Rumary and Thorpe, 1984).

Combinations of cytokinins and auxins at bud induction stage are usually not effective. The use of 10μM BA in combination with low concentrations of IBA at 10, 0.1, and 1μM, during bud induction in cotyledons of *P. canariensis* did not significantly increase bud production, but more importantly led to callus formation (Pulido *et al.*, 1990). Results showing identical trend as above for combinations of 10μM BA and NAA at 10 nM, 0.1 μM, and 1μM levels were reported for *P. coulteri* (Berlyn and Beck, 1980), *P. contorta* (von Arnold and Eriksson, 1981), *P. caribeae* (Webb and Diaz Santiago, 1983), and *P. rigida* (Patel and Thorpe, 1986).

The duration of exposure of explants to cytokinins is also critical. When various embryonic explants of *P. contorta* were employed, the differentiation of bud primordia and their subsequent development was strongly influenced by the length of time of exposure to BA, with a longer exposure of this cytokinin promoting callus formation and stunted growth of the buds (Patel and Thorpe, 1986). Exposure for shorter periods of time resulted only in the development of the primary shoots in epicotyl explants of black and white spruce (Rumary and Thorpe, 1984).

The ability of various carbohydrates to support growth was reviewed by Maretzki *et al.*,(1974) and their conclusion was that sucrose or its component monosaccharides resulted in the best growth in most plant cell cultures. They added that other carbohydrates can, however, be substituted for sucrose, and that growth responses differ, depending on the species or clone, but generally not the tissue from which the explant was isolated (cited in Dougall, 1972). Sucrose is the most common source of carbon employed for *in vitro* mass propagation of conifers, and it is a critical component for almost all the stages of the *de novo* organ formation (Thompson and THorpe, 1987). In a study to determine the best concentration of sucrose for induction and development of buds in cotyledonary explants of *P. canariensis,* Pulido *et al.* (1990) found that from 1, 2, 3, 4 and 5% tested, the optimum concentration for each stage was 3%. This same concentration was also found to be optimal for *T. occidentalis* (Harry *et al.*, 1987), however for *Pinus radiata* (Aitken-Christie and Thorpe, 1984), *Picea glauca* and *Picea mariana* (Rumary and Thorpe, 1984), and *Pinus rigida* (Patel *et al.*, 1986) sucrose concentration between 1 or 2% was found to be the optimum.

Most plant tissue culture media contain members of the B group of vitamins, such as thiamine, nicotinic acid, pyrodoxine, and pantothenic acid. In many cases, these vitamins are added to insure that a defficiency does not occur rather than on the basis of demonstrated need (Dougall, 1972). In many ways, the lack of requirement for vitamins in cell cultures is not surprising because whole plants are autotrophic for vitamins. This presumably means that they have the genetic information for the synthesis of these compounds, therefore the advantage of providing these compounds in plant tissue culture media may lie in minimizing stress on explants or cultures. The stress may result from a low-expressed capacity to synthesize vitamins, leakage of vitamins into the medium, or general stress due to a transfer to fresh medium which has not been conditioned (Dougall, 1972). The effect of vitamins is rarely reported in tissue culture of conifers, but in other species it has been reported sometimes as a critical factor for differentiation. For instance, Ohira *et al.*,(1976) pointed out that, in their study, there were some cultures showing low level of differentiation, i.e., peanut did not need exogenous thiamine, while those showing no capacity for differentiation, i.e., soybean, tobacco, and rice, required exogenous thiamine. Matsumoto *et al.*, (1976) showed that, in the absence of any vitamin, their tobacco crown gall tissue would grow to approximately one half the settled volume of their best medium, and required twice the time period to do so (cited in Dougall, 1972).

Adventitious bud development and elongation normally require the transfer of explants to a phytohormone-free medium. Reduction of the strength of the basic medium or sucrose normally promotes shoot elongation (Thorpe and Patel, 1984; Biondi and Thorpe, 1982). Shoot elogantion in *P. englemannii* was stimulated by reducing the concentrations of salts of AE (von Arnold and Eriksson, 1981) medium (Patel and Thorpe, 1986). In *T. occidentalis* (Harris *et al.*, 1987), maximal bud elongation and shoot development occurred on half-strength QP (Quoirin and Le Poivre, 1977) salt formulation, while for *L. occidentalis,* elongation of the shoots was improved when explants were transferred from half-strength QP medium during bud induction to half-strength SH (Shenk and Hildebrandt, 1972) during elongation (Harry *et al.*, 1991).

Inclusion of activated charcoal in the developmental medium improved elongation of the adventitious shoots (von Arnold and Eriksson; Harry *et al.*, 1987; Pulido *et al.*, 1990), and increased the number of buds produced on explants considerably (Patel *et al.*, 1986). Conifer-derived activated charcoal enhanced shoot elongation and increased secondary shoot formation in *P. glauca* and *P. mariana.* Other types of charcoal such as bone, or coconut-derived charcoal were not effective in causing such an effect (Rumary and Thorpe, 1984).

The stage known as multiplication has received recently more attention due to its importance in mass propagation of the initial adventitious shoots generated. Despite its importance, this stage has failed in many *in vitro* protocols for the genus *Abies* (Bonga, 1977; Saravitz *et al.*, 1987; Zygmont and Scharwz, 1987; Saravitz *et al.*, 1991). Harry *et al.*, (1987) have reported that although the number of shoots produced *in vitro* by embryonic explants of *T. occidentalis* was fairly low, secondary multiplication of the primary shoots was readily accomplished. Spontaneous axillary branching occurred after 4 months and the process could be enhanced by the addition of 1μM BA during one month of exposure. The average number of axillary buds produced from 6- to 7-month-old shoots was about 20 per shoot, and within one year, 100 shoots could be obtained from each primary shoot generated by the embryonic explant. One seed could generate between 200-400 shoots per year (Harry *et al.*, 1987). This example demonstrates the importance of shoot multiplication stage in the process of mass propagation *in vitro*. In *L. occidentalis*, when 1-year-old shoots were treated with 0.1μM 2iP, about 70% of the shoots produced 2-5 axillary shoots, but others auxins applied singly or in combinations, failed to induce axillary buds, and instead, the shoots turned brownish and died (Harry *et al.*, 1991). The best approach for shoot multiplication in *P. canariensis* was obtained when the terminal apical meristem was decapitated (Pulido *et al.*, 1990). This technique allowed the development of axillary buds normally inhibited by the apical shoot.

In general, rooting in conifers is a slow process and a problematic procedure when compared with rooting of herbaceous plants. Indeed it is the most difficult phase of the multi-organogenetic process (Thorpe and Biondi, 1984; Mohammed and Vidaver, 1988). Exogenous auxin applications and a reduction in minerals and sucrose are normally required for successful rooting (Rumary and Thorpe, 1984). A lower rooting temperature was found to be necessary for *P. menziesii* shoots (Cheng and Voqui, 1977). Agar-solidified medium has been a commonly used substrate for rooting, but frequently the roots produced in this system fail to survive under field conditions. Agar probably impedes gas exchange as well as the production of root hairs, and the option of employing substrates like peat, vermiculite, or perlite is preferable (Mohamed and Vidaver, 1988; Pulido *et al.*, 1990). In contrast to most coniferous species (Mott, 1981), rooting process of *in vitro* cedar shoots was easily responsive, and the best *in vitro* rooting treatment was achieved when the shoots were cultivated in half-strength QP medium with 0.1 mM IBA for 9 days and then transferred to Redi-Earth®. Under these conditions about 60% of the shoots formed roots within 4 to 6 weeks (Harry *et al.*, 1987). The optimum medium for inducing adventitious roots in *P. canariensis* was when liquid-pulse treatment was performed employing 1 mM IBA for 4 hours, and then transferrring the shoots to a peat:vermiculite (1:1) substrate moistened with one-quarter strength MCM medium. Using this treatment, survival rates of 90-100% was not uncommon (Pulido *et al.*, 1990). Rooting treatments were ineffective for *A. fraseri,* probably due to the size and lack of uniformity in the shoots (Saravitz *et al.*, 1991).

Embryogenesis

In vitro embryogenesis of conifers is a process analogous to zygotic embryogenesis, but one in which a single cell or a small group of vegetative or somatic cells are the precursors of the embryos (Ammirato, 1983). Unlike organogenesis, somatic embryogenesis can reproduce events of zygotic embryogenesis with the production of embryos presenting a bipolar structure. Somatic embryogenesis also provides an ideal experimental process for investigations of plant differentiation and the mechanisms of expression of totipotency in plant cells (Thorpe, 1988). Haberlandt postulated that any plant cell, given the appropriate stimuli and ideal conditions, could be induced to regenerate entire plants (cited by Brown and Thorpe, 1986).

In the normal zygotic process, embryogenesis is produced either by a process known as simple polyembryony as for example in *P. glauca* (Owens and Molder, 1984a) or by a different process called cleavage polyembryony as it happens in *Pinus contorta*

(Owens and Molder, 1984b). The development of a method of embryogenesis *in vitro* for conifers is a significant breakthrough, since besides the potential of mass propagation, it also provides a threshold for biotechnology research in protoplast cultures and genetic transformation (Dunstan, 1988). Somatic embryogenesis in conifers has so far been demonstrated to happen in tissues that proliferate when the zygotic embryo is placed in the tissue culture environment (Dunstan, 1988). This process is somehow similar to organogenesis, where adventitious buds arise from proliferating totipotent cells coming from seed tissue. However, for somatic embryogenesis a callus like tissue is usually formed in the presence of a high auxin to cytokinin ratio, whereas for bud induction no callus usually appears, since cytokinins singly or in combinations are employed or a low auxin to cytokinin ratio is used (Dunstan, 1988).

For many years, considerable effort has been directed to the establishment of developmental patterns for somatic embryogenesis protocols in conifers (Durzan and Steward, 1968; Chalupa and Durzan, 1973; Chalupa et al., 1976; Durzan and Chalupa 1976a; 1976b; 1976c; 1976d; Durzan et al. 1980). A polarized embryo-like structure was observed which sometimes contained suspensor-like cells, in *Pinus banksiana* (Durzan and Steward, 1968; Chalupa *et al.*, 1976; Durzan and Chalupa 1976a), *Picea glauca* (Durzan and Steward, 1968), *P. abies* (Chalupa an Durzan, 1973), and *Pseudotsuga menziesii* (Durzan, 1980). These structures were referred to as sphaeroblasts.

The first recognizable stage of somatic structures resembling embryos *in vitro* has been characterized as translucent group of cells containing long vacuolated suspensor-like cells subtending smaller dense meristematic cells of the embryonal apex (Hakman *et al.*, 1985). This configuration has been generally incorrectedly termed callus, but because this tissue is composed of organized structures, the term embryogenic tissue should be more appropriate (Tautorus *et al.*, 1991). The development of somatic embryogenic cultures and the regeneration of germinated somatic embryos into plantlets was first reported for *P. abies* (Hakman *et al.*, 1985), where the choice of the explant, composed mainly of immature embryos played a very special role. In this first report, immature and mature zygotic embryos were cultured in presence of high concentrations of 2,4-D (10-20μM) in combination with 5 μM of BA. After a few days the inoculum turned into a whitish, translucent tissue at frequency of 38% for immature embryos and 8% for matured explants. More recently, a higher concentration of auxin, usually 2,4-D, has been employed in most attempts to generate embryogenic tissue. Protocols have been developed for others species of conifers such as *A. alba* (Schuller *et al.*, 1989), *A. nordmanniana* (Nørgaard and Krogstrup, 1991), *L. decidua* (Nagmani and Bonga, 1985; von Aderkas and Bonga, 1988a) *L. decidua x L. leptolepis, Larix leptolepis* and *L. leptolepis x L. decidua* (von Aderkas *et al.*, 1990; Klimaszewska, 1989a), *P. abies* (Chalupa, 1985), *P. glauca* (Hakman and Fowke, 1987a; Lu and Thorpe, 1987; Dunstan *et al.,* 1988; Hakman and von Arnold, 1988; Tremblay, 1990; Lelu and Borman, 1990), *P. mariana* (Hakman and Fowke, 1987b; Tautorus *et al.*, 1990a; Attree *et al.*, 1990b; Lelu and Borman, 1990), *Picea rubens*(Harry and Thorpe, 1991), *P. sitchensis* (Krogstrup *et al.,* 1988), *Pinus wilsonii* (Ying-hog and Zhong-shen, 1990), *P. caribeae* (Lainé and David, 1990), *P. elliottii* (Jain et al., 1989), *P. lambertiana* (Gupta and Durzan, 1986d), *P. serotina* (Becwar *et al.,* 1988), *P. strobus* (Finer *et al.*,1989), *P. taeda* (Gupta and Durzan, 1987a), *Pseudotsuga menziesii* (Durzan and Gupta, 1987) and *Sequoia sempervirens* (Bourgkard and Favre, 1988). For these various species, a key factor to success has been the choice of the explant. Megagametophytes, immature and mature zygotic embryos, young seedlings, and re-induced cotyledonary somatic embryos have been the source of responsive explants that initiated the process of somatic embryogenesis.

Adventitious Bud Developmental Anatomy

Cheah and Cheng (1978), using Douglas fir cotyledonary explants, reported the sequential stages of adventitious bud development by histological examination. The mode of cell activation and the pattern of cellular growth of bud culture prior to the appearance of meristemoid structure was compared in bud-callus culture and callus culture. This systematic analysis provided the identification of four anatomical features, namely meristemoids, bud primordia, shoot apex with needle primordia and adventitious buds. Yeung *et al.*, (1981) also examined histological events associated with shoot primordium formation in cultured *P. radiata* and found that organized structures took place in the epidermal and sub-epidermal cells in contact with the media, and that the initiation of this process began within the first 24 hours in culture. They also observed that the pattern of cell division was originally random, but soon became restricted to the lower epidermis and sub-layers of mesophyll parenchyma. These mitotic divisions appeared in a periclinal direction in cotyledonary explants in contact with cytokinin, while only anticlinal cell divisions were observed in the control (BAP-free medium). Later (Villalobos *et al.*, 1985) cultivated cotyledonary explants of radiata pine in the presence or absence of 10μM of BA, from day 0 to day 21, and presented two distinct developmental phases in the process of *de novo* organ formation for this system. The first phase presented events associated with induction and differentiation of the cells located mainly at the epidermis and first and second mesophyllic layers. This stage starts with the first periclinal division of cells and ends with the differentiation of promeristemoids, (an organized cluster of 6-8 cells of rounded shape) by 5 days of culture in cytokinin containing medium. Anatomically, four interrelated characteristics were described in this stage, as 1) increased mitotic activity; 2) restriction of intercellular space development; 3) suppression of stomatal complex formation; and 4) differentiation of promeristemoids. The second phase shows events related with further development of the initial structures formed during the *de novo* process. The control, cultivated in a medium void of cytokinin, developed normally and by the end of the treatment presented normal features such as presence of stomata complex, mesophyll parenchyma, spongy parenchyma and vascular bundles.

Biondi and Thorpe (1982), investigating the *de novo* organ formation process promoted in cotyledonary explants of *P. radiata,* observed events related to lipids, proteins, soluble N, free sugars and starch during the initial 3 weeks in culture. From their investigation, it was observed that very active metabolism occurred in the explants between days 0-3 in culture. This was reflected by the rapid and pronounced depletion of major reserve substances (lipids and free sugars) and a high initial respiration rate.

The information collected so far from the different morphogenetic systems described in the literature can be summarized into the following items: 1) that growth regulators are an absolute requirement for the *de novo* process, and 2) cotyledonary explants have become the most adopted source of inoculum for the organogenetic pathway due to its special characteristics; although some firs have presented some exceptions in utilizing that explant, that 3) there is a necessity for additional energy source, as well as optimum conditions of major salts and other medium components, since high metabolic rates occurred during differentiation and organ formation. It is also well demonstrated that *de novo* process appears after few hours in the inductive medium and is observed in parenchymatic cells, especially those near the epidermis cells.

Adventitious Root Developmental Anatomy

There have been few reported histological studies of adventitious root formation in plantlets from cotyledonary explants in conifers. Several authors have included an anatomical description of the shoots they obtained from coniferous material *in vitro*, such as *Cryptomeria japonica* (Ishikawa, 1974), *P. palustris* (Sommer *et al.*, 1975), *P. glauca* (Campbell and Durzan, 1975), *P. taeda* (Mott *et al.*, 1977), *P. menziesii* (Cheah and Cheng, 1978), *P. abies* (von Arnold and Eriksson, 1978), *P. radiata* system (Yeung *et al.*, 1981; Villalobos *et al.*, 1985). These histological and histochemical studies have been conducted mainly to present changes leading to initiation and development of adventitious shoots or to present an overview of the vascular connection in the root-shoot junction of tissue culture-derived plantlets and seedlings (Bender *et al.*, 1987). However, very few examinations of the early events leading to the cellular changes involved in adventitious root initiation have been reported, e.g. as in*P. glauca* and *P. mariana* (Patel *et al.*, 1986). Until now most of the understanding of root initiation leading to *in vitro* plantlet formation is superficial and based mainly on observations of seedling cuttings (e.g., Heaman and Owens, 1972; Smith and Thorpe, 1975; Montain *et al.*,1983; Gronroos and von Arnold, 1985).

Preliminary results of work with hypocotyls and rooted cuttings have shown that adventitious roots arise from diverse functioning tissues, and this is generally the case when callus appears at the base of a cutting following excision. Studies done by Reines and McAlpine (1959), Mergen and Simpson (1964), Kummerow (1969), and Heaman and Owens (1972) indicate that root formation at the base of cuttings does not differ much from lateral root formation in primary roots, where the central cylinder and the cortex play important roles in the generation of basal callus. The most common feature shows callus formation at the base of a cutting followed by the formation of tracheidal strands derived from the shoot cambium. Subsequent cell division in the callus gives rise to primordia and further development of tracheidal strands connection ultimately form the new central cylinder of the new primordia (Smith, 1975). The vascular connection of the new adventitious root formed in plantlets normally is less smooth than normal roots in seedlings, therefore the efficiency of uptake and translocation of minerals will be dependent to a large extent on the nature of this junction of the *in vitro* produced plantlets (Patel *et al.*, 1986; Bender *et al.*, 1987). Further development of root apical meristematic cells and subsequent elongation give rise to fully developed adventitious root formation.

A repeated feature of adventitious root formation in a wide variety of plant species is that initiation usually takes place in the vicinity of differentiating vascular tissues of the organ from which the root arises (Esau, 1965; Hartmann and Kester, 1968; Hassig, 1972). Root initials have been reported to arise from many different tissues, such as the phloem region, cambial zone, interfascicular parenchyma and vascular ray (Esau, 1965;

Smith, 1975). It seems that the point of origin of a root initial varies between species, and even within the same plant (Wilcox, 1955).

Patel *et al.*, (1986) studed adventitious root formation under the influence of IBA on the *in vitro*-formed shoots of *P. glauca* and *P. mariana.* They described swelling occurring at the base of the shoot, and some callus being formed below the base. Some of the cells within the base of the shoot and in the vicinity of the vascular system differentiated into cambium-like cells which later produced tracheid nests and resin canals. The tracheid nests were composed of irregularly arranged tracheids of various sizes surrounded by cells of the cambium. Some of the derivatives of the cambial cells located at the periphery of the tracheid nests differentiated into the root meristemoids, whose cells were small and contained densely cytoplasmic contents and large nuclei. Later on, these cells differentiated into the root primordia which then assumed the normal configuration of a root.

The studies reviewed in this chapter provides a strong evidence *per se* that all the stages of micropropagation *in vitro* are controlled to a certain degree by the various components of the culture medium and the environment. It is clear that until now the *de novo* morphogenetic process is based heavily on empirical processes of trial and error with some background on previous works. Although many biochemical studies have been complementing studies conducted on this field, still the process of *de novo* organ formation is not understood completely. It is my personal view that a better understanding will be achieved when all possible tools of modern biology are utilized in studies concentrating on the first hours of those single cells submitted to shoot and non-shoot formation.

III. MATERIALS AND METHODS

1. Plant Material and Culture Conditions

Seeds of *Abies amabilis* (Pacific silver fir) used in this study were obtained through the courtesy of the Tree Seed Centre, Surrey, Ministry of Forests, Province of British Columbia. These seeds were collected from open pollinated trees in two natural stands located in British Columbia. The location of these stands are as follows:

Seedlot #	Latitude	Longitude	Elevation	Location
04226	50°20'	125°32'	1150 m	Knight Inlet
04244	51°48'	127°26'	200 m	Milton River

Upon receipt at the University of Calgary, seeds were stored in polyethylene bags kept inside plastic containers, allowing minimum oxygenation under low temperatures (between 3-5°C). These seed lots served as the source of inoculum for all experiments referred to in this study. Seeds were initially submitted to different treatments with sulfuric acid (99.9% Fisher®) in a glass beaker. These treatments were intended both for scarification of the seed coat to improve further germination, and also to remove the resin storage from the seed coat. This treatment was found to be a potential sterilant, since without its application the seeds had a heavy presence of contaminants, especially fungi. After treatment, the beaker was covered with cheesecloth, and the seeds were washed under running tap water to remove the sulfuric acid, as well as any debris from the reaction of the acid with the seed coat. After washing for one hour, the seeds received a 15 minutes treatment employing commercial bleach (6% NaOCl) containing three to four drops of Tween® per 100 ml and were rinsed again in running cold tap water and left in this condition for 2 days for hydration. This latter procedure was necessary since even after the sulfuric acid treatment and subsequent rinsing, the seeds contained dark debris and resin located throughout the seed coat. Following this period of hydration, the tiny seeds and those that floated in water were discarded along with those that possessed additional defects like broken seed coat, presence of necrotic tissue, etc.

Following hydration, the viable seeds were resterilized under sterile conditions in a laminar air flow cabinet. These treatments consisted of the application of 30% commercial bleach containing three to four drops of Tween® per 100 ml for 15 minutes, followed by four 5-min rinses with sterile double distilled water, after which the seeds were submitted to a solution of 0.1% mercuric chloride (w/v) for 3 minutes and again rinsed thoroughly with double distilled water. After this final step, the de-contaminated seeds were put into sterile plastic Petri dishes (90 x 15 mm) which were sealed and stored under dark and low temperature (2°C to 5°C) conditions and tested for various time periods.

For explants, the seed coats and megagametophytes were removed aseptically from stratified seeds and the naked embryos were plated horizontally on medium containing 1% sucrose and gelled with 0.7% Difco agar®. The pH was adjusted to 5.7-5.8 before autoclaving at 121°C under 15 atm. for 15 min, after which it was poured (25-30 ml) in sterile plastic Petri dishes (90x15 mm) and then allowed to solidify under aseptic conditions in a laminar air flow cabinet. Initially the explants (entire naked embryos) were incubated at 2°C to 5°C under dark conditions for different time periods and then transferred to a growth chamber at 27±1°C in a 16-h photoperiod provided by Sylvania Gro-Lux® F40712 Gro-WS lights. Photon flux rate was maintained at 80-100 μmol.m^2.s^1. After five days, the viable firm, undamaged, and naked embryos were divided into cotyledons (at the cotyledonary node), hypocotyls, epicotyls and radicles. The number of cotyledons per seed varied from 3 to 7. All these explants were used as source of inoculum, but in a few initial experiments whole embryos were also employed.

2. Organogenesis

In general, the process of plantlet formation via organogenesis requires critically assessment of four distinct stages: (i) induction of shoot buds, (ii) development and multiplication of these buds, (iii) rooting of the shoots, and (iv) hardening the plantlets

(Thorpe and Patel, 1984). The following items describe the experimental approaches designed to achieve each one of these stages.

2.1. Bud Induction and Elongation

The optimum conditions for each factor presenting some influence on the formation of adventitious buds was determined experimentally and tested under different conditions. This methodology was adopted for each critical stage in the development of organogenesis. After submitting the explants for periods of time in direct physical contact with single or combinations of phytohormone-containing medium, the explants were transferred to a basal medium without any growth regulators. All the experiments were repeated at least twice, with a minimum number of three replications per experiment. Each replication had approximately 37 to 39 cotyledons, as an average, and the cotyledons were randomly distributed throughout all experiments, to conform to one of the assumptions for application of ANOVA.

2.1.1. Selection of Embryonic Explant

Cotyledonary explants ranging in size from 3.5-8.5mm and 0-12 days old were tested under combination of cytokinins for the first two weeks and then transferred to a nutrient medium lacking phytohormones. The experiments for this section were done keeping standard procedures as described in section of plant material and culture conditions, and were designed to optimize results attained previously. An average of 37 cotyledonary explants were plated in each Petri dish.

The effect of size and age of hypocotyl and epicotyl explants were also determined. In this case, 5,10 and 15 mm explants taken at 0, 7, 14, 21, 28 and 35 days were tested under identical methods as described for cotyledonary explants above.

2.1.2. Influence of Stratification on Embryo Vigor

The working hypothesis for designing this experiment was to determine the possible implications of cold stratification on adventitious shoot formation and shoot vigor in Pacific silver fir. The basic assumption was that stratification increases the embryos' vigor, and consequently could have a possible role in enhancing adventitious shoot production and shoot quality. In order to test this hypothesis, an experiment was designed with ten replications for three different treatments. Each treatment had an average of 36 cotyledonary explants per replication and were repeated twice.

The three approaches consisted of seeds submitted to the following treatments: I) no stratification; II) one week under stratification, and III) five weeks of stratification. The experiment consisted of putting whole imbibed sterilized seeds inside a plastic container. This container was sealed with Stretch'n Seal®, and kept at temperature (2°C to 5°C) in complete darkness for 0 to 35 days. At the end of this period, the embryos were then dissected out using the previously described procedure and germinated for cotyledonary explants. The explants were placed on a medium containing 10μM BA for the first and transferred to identical equimolar concentration of BA plus zeatin for the second. At the end of the period of exposure in phytohormones, the inoculum were subcultured to a half-strength medium, being then subcultured bi-weekly until 12-weeks old when the experiment was statistically evaluated.

2.1.3. Nutrient Medium

Six basal media were tested both at full- and half-strength for all the stages of *in vitro* mass propagation. These included: SH (Schenk and Hildebrandt, 1972, as modified by Reilly and Washer, 1977), MCM (Bornman, 1983), QP (Quoirin and LePoivre, 1977), MS (Murashige and Skoog, 1962), DCR (Gupta and Durzan, 1985), and AE (von Arnold and Erikson, 1981). For half-strength nutrient medium concentration, only half of the recommended major salts were used.

Hypocotyl, epicotyl, radicle, whole embryos and cotyledonary explants of specific age arising from previous experiments were employed as sources of inoculum. Each treatment had five replications repeated two times and approximately 36 cotyledons and/or 9 hypocotyls, radicles, epicotyls and whole embryos explants were used per replication. After transferring the inoculum to half-strength medium without phytohormones, it was subcultured every two weeks, until the third month when evaluation and statistical analyses were done.

2.1.4. Phytohormone(s) Determination

Using specific nutrient medium and source of explants as ascertaind previously to be the most responsive, phytohormone(s) requirements were then determined. The influence of different cytokinins and auxins applied alone or in combination were then determined. The phytohormones zeatin, kinetin, BA, 2,4-D and NAA, were tested alone or in specific combinations. These combinations were applied for a specific period of time as found out experimentally to be the optimum, and followed or preceded the application of a single cytokinin or auxin. After these initial sets of single and combinations of two phytohormones were interpreted, further experiments were conducted to test the effects of combinations of three cytokinins (BA+Z+K) on adventitious bud development. In this specific experiment, BA was maintained as first at 10μM for the first seven days, and kinetin as a second at the levels of 0.1, 1 and 10μM for the similar period of time. Zeatin at 0, 1 and 10μM were tested concomitantly as second for each level of kinetin, or alone as a third until completing 21 days in presence of phytohormones. The time of exposure in phytohormones ranged from one to three weeks and all the concentrations tested were restricted to 10μM, 1μM, and 0.1μM levels. At the time the exposure in phytohormones had been completed, the cotyledonary explants were then transferred to a half-strength medium. They were subcultured each two weeks, until they were three months old, when the evaluation and statistical procedures were applied.

2.1.5. Time of Exposure in Cytokinins

To determine the length of period needed for maximum bud induction and development, cotyledonary explants were plated in a medium containing the best combinations of cytokinins determined previously for a period of 7, 14 and 21 days, and then transferred to a half-strength medium without cytokinins to allow bud elongation. Subcultures were performed each two weeks until final evaluation after 10-weeks. Each treatment had three replications, with an average of 36 cotyledons per Petri dish.

2.1.6. Gelling Agents

The following concentrations of gelling agents were tested using hypocotyl, epicotyl and cotyledonary explants: Gelrite® at 0.2%, 0.25%, 0.3%, and 0.35%, and Noble agar® or Bacto-Agar® at 1%, 0.9%, 0.8%, 0.7% and 0.6%. All these treatments were tested using a single cytokinin concentration supplemented to a nutrient medium for the first week and a combination of cytokinins applied for the second week. Besides the differences in concentrations of the gelling agents, all the standard conditions were maintained (see plant materials and culture conditions). Four replications and two repetitions were accomplished for this experiment.

2.1.7. Sucrose

Five concentrations of sucrose (30, 60, 90, 120, and 150 mM) were compared in relation to BFC and SFC. These concentrations were kept constant during the first two stages of each experiment and were maintained at 60mM, after transferring the explants for elongation in a medium lacking cytokinins. This experiment was designed using optimum cytokinin concentrations determined previously, and finally the explants were transferred to a medium lacking phytohormones. An average of 345 cotyledons (38 to 39 cotyledons per Petri dish) were employed per treatment in a total of the nine replications. This experiment was repeated twice.

2.1.8. Vitamins

Six concentrations of SH vitamins were tested in this experiment (1×, 2×,..,6×). These concentrations were maintained constant during the first two stages of each experiment but were changed after transferring the explants to a half-strength SH medium void of phytohormones. In this case, elongation medium was complemented with the normal prescibed dose of vitamin and thus maintained for all subsequent subcultures, until statistical evaluation was performed. The phytohormones levels and form of application were maintained as for testing the levels of sucrose. For this experiment, an average number of 310 cotyledons were used per treatment, where eight replications were repeated two times.

2.1.9. Effect of "Pulsing" in Cytokinin

Whole embryos, epicotyl, hypocotyl and cotyledonary explants were immersed for 1, 2, 3, and 4 hours in filter-sterilized 1mM BA aqueous solution with the pH adjusted to 5.0. Following this "pulsing" treatment in high concentration of cytokinin, the explants were then plated horizontally in a medium with or without phytohormones keeping the standard procedures outlined before in the introduction of this chapter. Single or combinations of cytokinins (BA and Z) were tested as first or second for the period of one week after the initial "pulsing" treatment. Following exposure in phytohormones for no longer than 14 days, the explants were then transferred to a free-cytokinin medium for bud elongation.

2.2. Shoot Elongation

This particular stage of the micropropagation of *A. amabilis* involved the development of the nodular tissue, formed during bud induction, into shoots with primary needles (Thorpe and Hasnain, 1988). Unless otherwise stated, this stage implied the transfer of isolated shoots approximately 10 to 12-weeks old, and >5mm high to a nutrient medium with low concentration of salts and without any phytohormone. The medium's pH was always adjusted initially at 5.7-5.8 and contained $0.5g.l^{-1}$ of activated charcoal (Sigma No. C4386) which was added prior to autoclaving. The percentage and quality of gelling agent did not vary from the one specified in standard procedures for bud induction. Explants were incubated at $27 \pm 1°C$ in a 16-photoperiod that was provided by Sylvania Gro-Lux® F40712 Gro-WS lights. Photon flux rate was maintained at 70-80 $\mu mol.m^{-2}.s^{-1}$. Sub-cultures were performed bi-weekly.

2.2.1. Effect of Salt and Sucrose Concentrations on Shoot Elongation

The basic assumption to be tested in this experiment was that lower concentrations of salt and sucrose would be optimum for plantlet elongation. Shoot explants, 10 to 12 weeks-old (explants ≥ 5mm) were selected and transferred to glass storage jars (100 x 80 mm) containing 120 ml of medium. These glass jars were covered with Petri dish lids and sealed with Parafilm®. Four media (SH, MCM, DCR and QP) were tested at full and half-strength salt concentration and were supplemented with 0 (control), 30, and 60 mM of sucrose. To test the effects of these different salt concentrations and sucrose levels on growth, a factorial 3x4 experiment was designed for both full- and half-strength nutrient medium. After 8 weeks in culture, the explants were measured and evaluated. To optimize shoot elongation, the two best media formulations (SH and DCR) were tested at full-, half-, one-third and one-quarter strength using the same sucrose concentrations. A 2x4 and 3x4 factorial designs were performed, and average heights were taken and analyzed after 10 weeks through an ANOVA.

2.3. Shoot Multiplication

In order to promote multiplication of shoots two approaches were tried: i) removal of the shoot apex and subsequent reculture of the decapitated explants and excised shoot tips onto a nutrient medium lacking phytohormones, and ii) induction of adventitious and axillary shoots through combinations of phytohormones. Unless otherwise stated, shoots 2.5 cm high were the major source of inoculum. These adventitious shoots were about 7 months-old, and came mainly from cotyledonary explants. The cultures were incubated at 24±1°C under a 16h-photoperiod and 80 μmol.m^{-2}.s^{-1} photosynthetically active radiation.

2.4. Adventitious Root Formation

For rooting experiments, 5 to 6 month-old adventitious shoots were used. Because the shoots were at various development stages, only those previously subjected to an elongation treatment for 8 weeks were selected. All elongated shoots older than 20-weeks with a distinct stem and about 10 mm high were selected.

After each initial rooting treatment the explants were planted out into glass jars containing vermiculite and sphagnum (1:2) supplemented with nutrient medium. The nutrient medium consisted of a quarter-strength SH plus 30 or 60 mM sucrose and standard dose of vitamins, which was added to the substrate for moisture as well as a source of nutrients and energy to the developing plantlets. After adjusting the pH at ±5.0, the medium was added to the substrate, and both were autoclaved at 120°C. The glass jars containing the rooting substrate were covered with sterile plastic lids and sealed with Parafilm® to allow gas circulation. The cultures were incubated in a special growth chamber that provided relative humidity outside the glass jars (±50%). A 16h-photoperiod was provided by fluorescent tubes that allowed an average light intensity of 80 μmol.m^{-2}.s^{-1}. The temperature was maintained at approximately 21:18°C (light:dark). In all experiments, rooting was assessed at approximately each 30 days by digging up and replanting the shoots. The percentage of rooting shoots was calculated as percent shoots rooted/ shoots under rooting treatment, as opposed to percent shoots rooted/ shoots surviving.

2.4.1. Effect of "Pulsing" on Hypocotyl Cuttings

In order to activate the rhizogenic potential of the hypocotyl cuttings, different approaches were tried in this experiment. These include the following:

i) pulsing the inoculum in 1mM IBA and or 1mM NAA for 1, 2, and 4 hours, and then transferring the explants to glass jars (100 x 80 mm) containing a mixture of vermiculite, ready-earth (Terralite®) and sand (1:1.5:1.5). To this soil substrate was added a quarter-strength medium supplemented with 30 mM of sucrose. The pH was adjusted to 5.0 before autoclaving for 20 minutes.

ii) transferring the explants directly after pulse treatment to glass jars containing a quarter-strength medium plus $7.0 g.l^{-1}$ Noble agar® and 30mM of sucrose, with the same pH as above. The explants were maintained under this condition for 5 weeks and then transplanted into a jar containing soil substrate like the former treatment;

iii) culturing the explants in a quarter-strength medium supplemented with sucrose and different concentrations of NAA for a period of 5 weeks, and then transferring them to a new sterilized soil substrate just containing distilled water.

2.4.2. Effect of "Pulsing" Adventitious Shoots

The bases of the shoots were immersed in filter-sterilized 10 mM IBA or 10mM NAA solutions for 1, 2, and 4 hours. The pH was adjusted to 4.5. Following this initial treatment the explants were placed into glass jars sealed with Parafilm® (100 x 80 mm) containing mixtures of vermiculite and sphagnum (1:1) with sterilized nutrient medium. Sucrose was maintained at 30μM.

2.4.3. Incubation with Low Concentrations of Auxins

Adventitious shoots were cultured in rooting substrates consisting of peat-perlite (1:1) and a nutrient medium, plus NAA and IBA used singly at 1μM, 5μM or 10μM. Application of NAA and IBA in combination at equimolar concentrations of 5μM or

10μM were also tested. The pH of the rooting medium was adjusted to 4.5, which was used to moisten the rooting substrate. Shoots were incubated for 6 weeks and then transferred to a rooting substrate just containing distilled water.

2.4.4. Effect of Combinations of Auxins and Sucrose

A 4x4 factorial experiment comparing effects of IBA levels (0, 1, 10, and 100μM) and sucrose concentrations (0, 30, 60, and 90mM) on rootingwas tested at first. The light conditions were maintained at 50-60 μmol.m^{-2}.s^{-1} from Gro-lux. All other conditions from the previous experiment were preserved, with the exception that after 4 weeks the explants were transferred to a medium lacking phytohormone, but with the same concentrations of sucrose. A second batch of experiments was also done using single or any possible combinations of two auxins like IAA, 2,4-D, IBA and NAA at 1μM, 10μM and 100μM. All the conditions of the former experiment were repeated with the exception that sucrose was maintained at a single level of 30μM.

3. Somatic Embryogenesis

Seeds of Pacific silver fir (*Abies amabilis* Dougl.) Forbes. were hydrated and disinfected following identical procedures as described in material and methods for organogenesis. *In vitro* germinated embryos were cultivated for a limited time under light conditions, at 80-100 μmol.m^2.s^1 and 24±1°C, respectively. The 16-h photoperiod was provided by Sylvania Gro-Lux® F40712 Gro-WS lights. After germination and culture for different periods in light, the explants were put under dark conditions to promote callus formation (see sections 3.1 to 3.3, p.42 and 43). Unless otherwise stated, the culture medium was always Schenk and Hildebrandt (1972) supplemented with 88 mM of sucrose, 7 g.l^{-1} (w/v) of Noble agar (Difco Bacto®-control 781971), 500 mg/L of casein hydrolysate, 450 g/L of L-glutamine and 100 g/L of myo-inositol. The culture medium was adjusted to 5.7 - 5.8 pH before autoclaving. Replications and randomization were

Table 1. Composition for six nutrient media, as indicated below: Schenk and Hildebrandt (SH), medium for conifer multiplication (MCM), Gupta and Durzan (DCR), Murashige and Skoog (MS), von Arnold and Eriksson (AE), and Quoirin and LePoivre medium (QP).

Salt formulation (mg/l)	**SH**[1]	**MCM**[2]	**DCR**[3]	**MS**[4]	**AE**[5]	**QP**[6]
Major Salts						
KNO_3	2500	2000	340	1900	1900	1800
$NH_4H_2PO_4$	300	-	-	-	-	-
$CaCl_2.2H_2O$	200	-	85	-	180	-
$MgSO_4.7H_2O$	400	250	370	370	370	-
$Ca(NO_3)_2.4H_2O$	-	500	556	-	-	1200
$(NH_4)_2SO_4$	-	400	-	-	-	-
KCL	-	150	-	-	-	-
Urea	-	150	-	-	-	-
KH_2PO_4	-	270	170	170	340	270
NH_4NO_3	-	-	400	1650	1200	400
$CaCl_2$	-	-	-	440	-	-
$Ca(NO_3)_2.4H_2O$	-	-	-	-	-	360
Minor salts						
H_3BO_3	5.0	1.5	6.2	6.20	0.63	6.2
$ZnSO_4.7H_2O$	1.0	3.0	8.6	8.6	-	8.6
$MnSO_4.H_2O$	10.0	0.17	22.3	22.3	2.20	1.0
$Na_2MoO_4.2H_2O$	0.1	0.25	-	0.25	0.025	0.25
KI	1.0	0.25	0.83	0.83	0.75	0.08
$CuSO_4.5H_2O$	0.2	0.025	0.25	0.025	0.0025	0.025
$CoCl_2.6H_2O$	0.1	0.025	0.025	0.025	0.0025	0.025
$NiCl_2$	-	-	0.025	-	-	-
$NaMoO_4$	-	-	0.25	-	-	-
Zn-EDTA	-	-	-	-	4.05	-
Iron						
$FeSO_4.7H_2O$	15.0	-	27.8	-	14.0	55.7
$Na_2EDTA.H_2O$	20.0	-	37.3	-	19.0	74.5
$Na_2FeEDTA\ 2H_2O$	-	37.5	-	40.0	-	-
Vitamins						
Thiamine-HCl	5.0	1.7	1.0	0.1	5.0	5.0

---

[1] Schenk, R.V., and Hildebrandt , A.C.(1972). Can. J. Bot. 50:199-204, as modified by Reilly and Washer (1977).

[2] Bornman, C.H. (1983). Physiol. Plant. 57: 5-16.

[3] Gupta, P.K.,and Durzan, D.J. (1985). Plant Cell Rep. 4:177-179.

[4] Murashige, T. and Skoog, F. (1962). Physiol. Plant. 15:473-496.

[5] von Arnold, S., and Eriksson, T. (1981). Can. J. Bot. 59:870-874.

[6] Quoirin, M., and LePoivre, P. (1977). Acta Hortic. 78:437-442.

Cont'd.

Salt formulation (mg/l)	**SH**[1]	**MCM**[2]	**DCR**[3]	**MS**[4]	**AE**[5]	**QP**[6]
Nicotinic acid	5.0	0.6	0.05	0.5	2.0	5.0
Pyrodoxine-HCl	0.5	1.2	0.5	0.5	1.0	0.5
Glycine	-	2.0	2.0	2.0	2.0	-
Pantothenate	-	0.5	-	-	-	-
Folic acid	-	1.1	-	-	-	-
Biotin	-	0.125	-	-	-	-
Myo-inositol	1000	90	200	100	100	100
Asparagine	-	-	-	-	-	100
Amino acid	-	-	-	-		-
L-Glutamine	-	-	-	-	0.40	-
L-Alanine	-	-	-	-	0.05	-
L-Cysteine-HCl	-	-	-	-	0.02	-
L-Arginine	-	-	-	-	0.01	-
L-Leucine	-	-	-	-	0.01	-
L-Phenylalanine	-	-	-	-	0.01	-
L-Tyrosine	-	-	-	-	0.01	-
L-Glycine	-	-	-	-	2.00	-

---

[1] Schenk, R.V., and Hildebrandt , A.C.(1972). Can. J. Bot. 50:199-204, as modified by Reilly and Washer (1977).

[2] Bornman, C.H. (1983). Physiol. Plant. 57: 5-16.

[3] Gupta, P.K.,and Durzan, D.J. (1985). Plant Cell Rep. 4:177-179.

[4] Murashige, T. and Skoog, F. (1962). Physiol. Plant. 15:473-496.

[5] von Arnold, S., and Eriksson, T. (1981). Can. J. Bot. 59:870-874.

[6] Quoirin, M., and LePoivre, P. (1977). Acta Hortic. 78:437-442.

used in all the experiments, each comprising of an average of 37 to 39 cotyledons per Petri dish, or 9 epicotyls or hypocotyls per Petri dish. The experiments were replicated at least five times and repeated twice.

3.1. Induction of Callus on Various Explants

After sterilization, the seeds were kept under low temperature (2°C to 5°C) for a period of 0, 7, 14, 21, 30, 60, 90 and 120 days. After these different periods the seed coat was dissected and the naked embryo was then divided into different segments. Explants such as whole embryos, radicle, hypocotyl, epicotyl and cotyledons were plated horizontally on Petri dishes containing 30 ml of induction medium, supplemented with a combination of either 9.1 μM 2,4-D and 4.5μM BA or 21.5 μM NAA and 0.45 μM BA. The cultures were incubated in the dark at 25±1°C. Subcultures were performed biweekly after the starting of callus formation. The necrotic tissues and phenolic substances that were subsequently formed in culture were removed constantly.

3.2. "Pulse Treatment" for Callus Induction

Pulsing treatment was carried out maintaining the same characteristics of the previous experiment (see item 3.1.) with the exception that after the initial pulsing treatment, the seeds were dissected and the naked embryos were cultivated in a germination medium containing only 30 mM of sucrose and 7 $g.l^{-1}$ Noble agar. Basically, the seeds were subjected to 12, 24, 48, 72 and 96 hours of exposure in liquid nutrient medium containing 0.1 mM 2,4-D plus 0.1μM BA or either 0.1 mM NAA plus 0.1μM BA, followed by seed dissection and cultivation of the excised embryos for 2 days in the dark under lower temperatures of 2° to 5°C and an additional 3 days under light conditions (80-100 $\mu mol.m^{-2}.s^{-1}$ and 24±1°C). After these initial procedures, the embryos were dissected further into 2, 3, 4 and 5mm of hypocotyl, epicotyl and cotyledonary explants. These explants were plated horizontally on medium containing 9.1 μM 2,4-D plus 4.5μM

BA or 21.5 µM NAA plus 0.45 µM BA and incubated under dark conditions. After the induction of callus after 5 to 6 weeks, the explants were subcultured on an identical medium every second week. The necrotic tissues and phenolic substances that were subsequently formed in culture was removed constantly.

3.3. Effect of Combinations of Phytohormones

Various experiments were also performed using Gupta and Durzan (1985) medium employing 6 g.l^{-1} bacto-agar (Difco®) and 88 mM sucrose. The pH was adjusted to 5.7-5.8 prior to autoclaving. Different combinations of auxins and cytokinins such as IAA, 2,4-D, NAA, IBA, BA, Z, K and 2iP were used, and these phytohormones were at concentrations of 1, 2.5, 5, 7.5, and 10µM. Again, various embryonic explants were tried, similar to those described in experiment 4.1. All cultures were incubated in the dark at 25±1°C. Subcultures were done on identical medium after the starting of callus formation. The necrotic tissues and phenolic substances that were subsequently formed in culture was removed constantly.

4. Histology and Histochemistry

Histological studies were performed essentially to determine the target areas responding to exogenous phytohormones, and to identify critical stages during the *de novo* process in organogenesis through sequential examination of the histological events in a temporal frame. Particular emphasis was made in the detection of early promeristemoid formation and its development into shoots. Also of importance was to gain some insights from the further development of adventitious roots. The type of vascular connection formed between roots and shoots during the stage of root development was determined through the number and quality of xylematic tissues.

Special effort was allocated to determine, if possible, the origin and development of root primordia. Finally, attempts were also made to establish whether the development of the epidermis and the first and second cell layers in contact with phytohormones that turned into meristematic tissue could be characterized by preferential synthesis of DNA and proteins. Information in the role of protein, and starch accumulation and/or depletion during the *de novo* process through histochemical approaches were also sought.

4.1. Plastic Embedding

The specimens were gently cleaned, sectioned and kept in vials placed in a small ice bath and then fixed using 2% formaldehyde and 2% glutaraldehyde buffered with 0.05 M phosphate buffer, at pH 6.8. The specimens were then vacuum infiltrated for 15 min. at 26 p.s.i. to remove the air existing within the tissues, and then kept at 4° C for 24 hours. The tissues were dehydrated using methyl cellosolve followed by two changes of absolute ethanol and embedded in LKB Historesin (70-2218-500 Historesin Embedding Kit®) according to the recommendation proposed by Yeung and Law (1987).

The LKB Historesin consists basically of three components: I) a basic resin or a glycol methacrylate monomer (GMA), II) the activator (benzoyl peroxide powder) and III) a hardener, being a derivative of barbituric acid containing dimethyl sulfoxide (DMSO). The embedding medium was prepared just prior to need and used immediately since polymerization begin as soon as the solution was prepared. This was done by adding the infiltration solution (50 ml basic resin/liquid + 0.5 g of activator), hardener and polyethylene glycol 400 in a proportion of 15:1:0.6 mililiters of each component. PEG 400 was added to the medium in order to produce ribbons, since GMA alone is known for its inability to produce continuous ribbons (Yeung and Law,1987).

The specimens were put in the lower depression of the mold tray and oriented after the addition of the embedding medium, and allowed to polymerize for 120 minutes at room temperature. After this time the specimen blocks were released, wiped and sectioned at 2-μm thick sections with glass knives employing a Reichert Autocut microtome.

Following slide preparation and staining, the glass slides were made permanent with DPX synthetic resin (BDH®).

4.2. Paraffin Embedding

For bulky specimens, the paraffin embedding method was used. The specimens were fixed using 2% formaldehyde and 2% glutaraldehyde buffered with 0.05 M phosphate buffer, at pH 6.8, following identical procedure for plastic embedding method. After absolute alcohol dehydration the specimens were transferred into the 100% *tert*-butyl step of the alcohol (TBA) series, culminating with a dehydration in 100% TBA. The specimens were then infiltrated with Paraplast plus according to the procedure described by Jensen (1962). Each step was performed maintaining a minimum period of 24 hours for each change. Finally the tissues were embedded in Paraplast, and sectioned at 7μm. Before the specimens were embedded into the melted paraffin they were once again vacuumed under a temperature of 60°C for 15min. at 26 p.s.i.. Paraffin embedding was accomplished using a paper embedding boat put on a surface of ice water to be cooled down. After this procedure was completed, and the blocks were completely hardened, the paper boats were peeled away from the paraffin blocks. Finally the paraffin blocks were mounted and trimmed onto plastic block holders or plastic stubs and sectioned in a AO Spencer 820 microtome.

4.3. Histological and Histochemistry Staining

Plastic sectioning was accomplished employing a retracting microtome and sections 2μm thick were obtained using glass knives at 5° to 7° clearance angles. The ribbons were collected and floated on distilled water at room temperature onto clean glass slides pretreated with adesive, and then were dried on hot plates at approximately 60°C (Pappas, 1971). All the slides used for mounting sections were precleaned by soaking overnight in 70% ethanol containing a few drops of 1N HCl and rinsed with distilled water. The cleaned slides were then dipped in an adhesive or subbing solution and placed to dry in a dust-free area following procedures described by Pappas (1971).

Paraffin sectioning were performed on a Spencer rotary microtome. Sections 7μm thick were obtained employing steel knives. The sections were stained with safranin-fast green (Yeung, personal communication), where nuclei, phenolic substances, cuticle, and lignified elements stained green. For histochemical visualization of total carbohydrate, sections were stained with the periodic acid-Schiff (PAS) procedure (Jensen, 1962; Yeung, 1984). Insoluble carbohydrates such as starch stained red. For histological visualization of proteins, sections were stained using amido black 10B where proteins stained blue (Jensen, 1962; Jensen and Fisher, 1968; Yeung, 1980). After staining, the slides were made permanent with DPX synthetic resin (BDH®). The specimen were examined and photographed using a Leitz Aristoplan microscope (Wild Leitz®). The image were recorded using Kodak Technical and Kodak Plus-X Pan film.

4.3.1. Promeristemoid Formation in Cotyledonary Explants

In order to study the emergence of pro-meristemoids, depletion and/ or accumulation of starch and proteins, and the establishment of adventitious shoots, combination of the best explant and medium conditions for shoot and non-shoot formation were employed. The cultured explants were then harvested at various stages of this treatment (namely, 0, 1, 2, 3,... and so on until day 22 in culture), dehydrated and fixed for plastic embedding. The same approach was used for the control, with the exception that the explants were cultivated for the same period in a half-strength SH medium lacking phytohormones. After completing all steps for plastic embedding, the specimens were sectioned at 2μm and stained in Feulgen, amido black 10B and Toluidine blue O (Yeung and Law, 1987; Yeung, 1984) and the glass slides were made permanent.

4.3.2. Apical Meristem Formation in Adventitous Shoots

Apical meristems from 5-month-old adventitious shoots and normal seedlings were harvested, fixed, embedded and sectioned at 2μm sections following the pattern for plastic embedding. The adventitious shoots were generated as described in the previous experiment using cytokinin alone and combinations of cytokinins for second as well as all optimum conditions determined experimentally for generation of adventitious shoots.

4.3.3. Vascular Connection Pattern in Adventitious Shoots

Adventitious roots from cotyledonary explants, and roots from seedlings were harvested and fixed following the pattern for paraffin and plastic embedding. For this study, tissues from 11-months old plantlets and seedlings were employed. Here again, the explants were obtained following the treatment indicated previously in item 4.3.2.

4.3.4. Adventitious Root Primordia Formation

In order to investigate the earlier events leading to adventitious root primordia, histological and histochemical studies were conducted on explants presenting various stage of adventitious root development. Every possible kind of rooting explant was selected, but emphasis was put on rooting of adventitious shoots while they were still attached to the original cotyledonary explant. These shoots were singular in the sense that, unlike others shoots with *in vivo* induced roots, they developed a sort of spontaneous adventitious roots without callus formation while the shoots were very young (≤ 5 mm high). Also these adventitious shoots coming from cotyledonary explants were attained through combinations of auxins and cytokinins (2,4-D and BA or NAA and BA) during the bud induction stage and probably represented a side effect of traces of auxins possibly still present in the tissues or vascular system of the original explant.

5. Data Collection and Analysis

During the induction of an organogenetic pathway for *A. amabilis*, culture characteristics and number of shoots were recorded at each experiment for a specific stage and a final count of shoots per cotyledon was made after two initial months, and then after each subsequent month following subcultures. Evaluation of treatment effects on survival and shoot production was based on four variables:

1) the percentage of shoot and/or root induction,

2) the number of shoots per cotyledon,

3) bud forming capacity (BFC) index, as a result of the average number of buds per cotyledon multiplied by a percentage of cotyledons forming buds divided by 100,

4) shoot elongation capacity (SEC) index, as a result of the number of shoots higher than 5 mm multiplied by a percentage of cotyledons forming buds divided by (100 x number of replications).

Variables 2, 3 were analyzed (Giselle Engels and Tak Fung - University of Calgary, personal communication) using Analysis of Variance (ANOVA). Additional analyses were applied to subsets of the data using one-way ANOVA. The chi-square Goodness of Fit with correction for continuity was also applied to stratification treatments to determine whether the data could conform to a certain theoretical distribution. Duncans' multiple range test with corrections for unequal numbers when apropriate was used to compare treatment means unless otherwise noted. A probability level of 0.05 was considered significant (Zar, 1984).

All hypotheses tested were assumed *a priori* before starting and measuring the results of each experiment. The exceptions are indicated and in each case were analyzed through specific statistical analysis. The results were interpreted based on the number of buds per explant, number of explants forming buds, BFC and SEC indexes in response to exposures to various cytokinins and auxins.

IV RESULTS AND INTERPRETATIONS

The initial approach for micropropagation protocol of *A. amabilis* was based on production of adventitious buds following procedures delineated by Thorpe and Patel (1984). Numerous experiments were run in parallel to provide some indication of what might be effective for achieving bud induction. The primary aim was to find out an explant that would respond and produce buds when cultivated under appropriate kinds and concentrations of phytohormones. Once this goal was achieved the subsequent steps would be further optimization of the best results attained. Embryonic explants cultivated under various salt concentrations such as MS, SH, MCM, AE, QP and DCR were tested in combination with auxins and cytokinins. Single cytokinins such as zeatin, kinetin, BA, and 2iP were tried at various levels from 0.1, to 50μM for 7 to 21 days in culture. Also auxins such IAA, NAA, 2,4-D and IBA were evaluated similarly. Gelling factors such as Gelrite® (0.2 to 0.35%) and Bacto-Agar® (0.6 to 1.0%) were tried with the various phytohormones described above. These initial attempts were generally unsuccessful over a period of 2 years, since most of these experiments failed to induce adventitious shoots capable of further development.

The most promising results arising from these preliminary experiments to induce organogenesis *in vitro* occurred when the explants in the presence of bud-forming medium started to swell and formed nodular structures. However, the first needle primordia rarely developed, and the few buds formed failed to undergo any further development and died following this stage. In a few cases when a limited number of buds turned into shoots, they became dormant, lost their green color and failed to elongate. From all these initial attempts 10μM BA appeared to be the best kind, and concentration of phytohormone, and cotyledons the best explant for generating nodules. These two factors were selected for further experimentation. Thus, the results presented in this dissertation reflect a limited number of selected experiments, with emphasis on those factors that have been shown to be critical for the *de novo* organogenetic pathway of plantlet formation in *A. amabilis*.

1. Establishment of an Aseptic Culture

Successful sterilization of explants is a requirement for *in vitro* culture, and often involves several steps (Thorpe and Patel, 1984). Seeds of *A. amabilis* possess large pockets of suface resin distributed throughout the seed coat which probably give them additional protection. These seeds can be stored under low temperature and moisture content for many years without significantly loosing their viability (Leadem, 1986). When the first attempts were made for seed sterilization a high percentage of fungal contamination usually appeared. This could be due to the presence of spores in a resistant form in or on the resin spots attached to the seed coat. These fungal spores were resistent to 30% bleach for 15 minutes after the seeds were soaked for 24 and 48 hours under running tap water. Because the concentration of hypochlorite required to desinfect Pacific silver fir seeds completely was close to lethal, these seeds were scarified briefly (1 to 5 min.) with concentrated sulfuric acid (99.9% H_2SO_4 Fisher®), followed by rapid dilution with a large volume of cold water. These methods decreased the percentage of contamination with no adverse effects on embryo viability, but this method did not eliminate the microorganisms completely. Also, scarification helped to remove the resin on seed coats and facilitated water imbibition and later dissection. Below is depicted the steps for the most effective sterilization treatment for this species. This regime was developed empirically over several months. This sequence was capable of eliminating the most common sources of infection for *in vitro* culture :

1) Leave the seeds at room temperature for 2hr after removal from storage
2) Immerse them in sulfuric acid (99.9%) for 3 minutes (30 ml H_2SO_4 per 90 grams of seeds)
3) Leave the seeds in running tap water to rinse for 30 minutes
4) Apply 30% commercial bleach (6% NaOCl) for 5 minutes just to remove the dark debris left by the sulfuric acid
5) Rinse and leave the seeds in running tap water for an additional 30 minutes

6) Hydrate the seeds in running tap water for 48 hours

Sterilization treatment in a laminar air flow cabinet:

7) Apply 30% commercial bleach (6% NaOCl) plus 3-4 drops of Tween per 100 ml for for 15 minutes.

8) Rinse four times with double distilled water

9) Immerse in 0.1% $HgCl_2$ solution for 3 minutes

10) Rinse thoroughly four times with double distilled water

11) Keep the seeds in plastic-sealed Petri dishes with low moisture content and under low temperature (2 to 5°C).

Although this treatment seems harsh, it did not have visible impact on seed or embryo viability, since the excised embryos germinated *in vitro* appeared healthy and normal (see fig. 2A and B, p. 64).

2. Organogenesis

In the formation of adventitious buds, there is an interplay between the inoculum, the medium, and the culture conditions. For optimum organogenesis, each of these components has to be critically assessed (Thorpe and Patel, 1984).

2.1. Selection of the Best Explant

Excised embryos of *A. amabilis* were non-pigmented and had an average of four (range of 3-7) closely appressed cotyledons. Of the embryonic explants tried, e.g., whole embryos, radicles, hypocotyls, epicotyls, megagametophytes and cotyledonary explants, only the latter demonstrated advantageous properties for *in vitro* manipulation. When whole embryos, hypocotyls and epicotyls were cultured on bud-induction medium, they swelled and a nodular tissue was observed proliferating from the cotyledons and sometimes from the hypocotyls in direct contact with the medium. However, considering that these explants have a large size, only a small portion of their surface area was in direct contact with the phytohormone-containing medium. This is also true for cotyledons still attached to the embryo; as only half of them were in direct contact with the medium.

The nodular tissues that developed in the epidermal layers of these explants did not elongate well and the whole inoculum turned a brownish color. With few exceptions, all the adventitious shoots that originated from these sources of inoculum died after a few weeks in the elongation medium. Attempts made to elongate those few tiny buds and shoots failed while they were still attached to the original explant. This fact, together with the question involving the surface area in contact with the medium, led to the conclusion that these explants did not constitute the best source of inoculum available for this experimental approach.

Another major problem in using whole embryos, hypocotyls and epicotyls as source of inoculum was that seeds of this species germinate asynchronously, making it difficult to ascertain the number of explants that would be available for experimentation at any given time. This asynchrony could be a reflection of general genetic diversity in the seeds. This heterogeneity was also reflected by the different sizes of seeds and by the number, size and vigor of the embryos and cotyledons. Also, a very large variability was found in germination (see Table 5, p. 62). Therefore, isolated cotyledons from *in vitro* germinated embryos were tried as the main source of explants. This system provided more explants, and the number of explants expected was more predictable. Using this inoculum the *de novo* organ process took place more synchronously, as a higher proportion of the tissue was in contact with the nutrient media. An added advantage in using this system was that cotyledonary explants could be selected individually by their size and general appearance. All these factors together made possible the selection of a more homogeneous population of explants, and reduced the variance of each experiment.

After experimentation, the best explant turned to be 5-day old embryos, germinated *in vitro* on sucrose (1%)-agar. These cotyledons were exposed to light for only

3 days (the time of exposure to light that will be used to designate the explant age in all experiments). Under these conditions the naked embryos underwent a process of maturation, with their hypocotyls turning into a reddish color and the cotyledons becoming firm and green. These combinations of nutritive conditions and light stratification are referred here as after germination medium.

Tables 2 and 3 illustrate the experimental methods used to test the aging effect on a specific type of embryonic explant on *de novo* organ formation. Cotyledonary explants of different sizes and ages showed differential response to the application of phytohormones and to the culture conditions as well. Cotyledonary explants generally formed buds on the entire length of the side in contact with the medium, but the bud forming capacity (BFC) and shoot elongation capacity (SEC) indexes were reduced gradually with increasing age and size. The developmental stage of the cotyledonary explants prior to culture greatly affected bud formation and shoot elongation potential. Cotyledons coming from embryos younger than 3 days old had not yet fully reached bud-forming capacity, while those older than 6 days had begun to lose their regenerative capacity, demonstrating that *A. amabilis* possess a narrow limit of response to *de novo* organ formation. These differences are also shown in Fig. 1A.

Table 3 shows the results of a 5x5 factorial experiment linking five salt formulations, as follows: MCM (Bornman, 1983),MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), DCR (Gupta and Durzan, 1985), and AE (von Arnold and Eriksson, 1981), applied to different ages of cotyledonary explants, from 2- to 6-day old. The results show that between days 3 to 4 the potential to generate buds is the highest when compared with days 5 and 6. After day 4 the capacity of bud formation decreases. Although day 8 explants produced the highest average number of buds per cotyledons, these buds did not elongate further. When Tables 2 and 3 are examined together a trend for capacity of bud induction is fairly visible, namely higher degree of response for 3- to 4-day-old explants. A close look at table 2 shows that by the time the cotyledons reached 9-days-old, the explants were capable of only forming small buds distributed throughout the length of the cotyledons than failed to elongate.

Figure 1.A. depicts an overview of 3-, 4-, 5-, until 11-day-old cotyledons after 6 weeks in culture on a SF medium. Table 2 also shows that the average number of buds per cotyledon and the overall percentage of cotyledons responding was higher in the 3-day-old explants relative to any other explant. Cotyledons younger than 3-day-old more frequently produced a smaller average number of shoots per cotyledons, but the percentage of cotyledons forming buds were normally higher than occurring in the 3-day-old. Also occasionally the number of buds elongating was higher for 2-day-old cotyledons, but the buds were not as healthy as those from 3-day-old cotyledons. Also many of these younger explants had a tendency to turn brownish and to die soon after the of bud elongation stage. When SEC indexes were compared for all ages of cotyledons, the SEC index was highest for 3-day-old cotyledons, surpassing by about 27% the second best result (Table 2).

2.2. Explant Responses to Stratification

Chilling method, seed source, collection year, germination temperature, and seed vigor all affect the germination response of *A. amabilis* to stratification, and it is apparent that stratification cannot be considered in isolation from other factors that interact directly or indirectly with the crucial events taking place during the chilling period (Leadem, 1986). The influence of cool temperature (± 2°C) and low content of moisture for certain period of time in promoting adventitious bud formation on cotyledonary explants of *A. amabilis* were also examined. Table 4 presents the results for the three periods of stratification treatments in which seeds of *A. amabilis* were submitted, namely: 0, 1 and 5 weeks. After these times under cold temperature, the seeds were dissected and the excised

Table 2. Effect of age of cotyledonary explants of *A. amabilis* on their bud forming capacity and shoot elongation capacity indexes.

Age of explant (days) *	Cotyledon length (mm)	%Cotyledon forming buds	No. of buds per cotyledon $X \pm SE$	BFC[1] index	SEC[2] >5 mm
3	3.5-4.0	68	3.14 ± 0.06^{a}	2.13 ± 0.16^{a}	14.1
4	4.0-5.0	55	$2.80 \pm 0.39^{a,c}$	$1.63 \pm 0.34^{a,b,c}$	10.3
5	4.5-5.0	63	$2.41 \pm 0.08^{a,b,c}$	$1.53 \pm 0.10^{b,c}$	8.2
6	4.5-6.0	58	$2.10 \pm 0.21^{b,c}$	$1.25 \pm 0.20^{b,c,d}$	6.8
7	5.0-7.0	48	$2.16 \pm 0.25^{b,c}$	$1.06 \pm 0.23^{c,d}$	4.4
8	5.5-7.5	41	4.31 ± 0.34^{d}	$2.01 \pm 0.52^{a,b}$	4.6
9	6.5-8.0	39	$2.64 \pm 0.27^{a,c}$	$1.01 \pm 0.06^{c,d}$	-
10	7.0-8.5	34	1.53 ± 0.11^{b}	0.52 ± 0.07^{d}	-
11	7.0-8.5	28	$1.75 \pm 0.44^{b,c}$	0.58 ± 0.22^{d}	-

Note: means followed by different letters are significantly different at $p \leq 0.05$ by Duncan Multiple Range Test.

(*) For all treatments, explants were maintained under conditions as follows:
age of cotyledon + SH[3] 10μM BA $\xrightarrow{7\text{ days}}$ SH 10μM Z + 10μM BA $\xrightarrow{7\text{ days}}$ ½ SH[4]

[1] BFC (bud forming capacity) index = (average number of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation performed after 8 weeks.

[2] SEC (shoot elongation capacity) index = (number of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications.

---

[3] Schenk and Hildebrandt (1972) nutrient medium

[4] Schenk and Hildebrandt (1972) half major salts concentration

Table 3. Effect of a 5x5 factorial experiment involving different salt concentrations and ages of cotyledonary explants of *A. amabilis* on bud forming capacity and shoot elongation capacity indexes.

Age of explant (days) and salt concentration *	Explant response (%)	No. of buds per cotyledon $X \pm SE$	BFC[1] index	SEC[2] >5 mm
MCM[3]				
day #2	65.7	1.93 ± 0.21^{a}	1.27	7.7
day #3	64.9	$1.71 \pm 0.25^{a,b}$	1.11	5.9
day #4	64.3	2.69 ± 0.33^{c}	1.73	10.3
day #5	26.3	$1.64 \pm 0.44^{a,b}$	0.43	3.1
day #6	34.2	3.20 ± 0.51^{d}	1.09	3.5
MS[4]				
day #2	29.6	1.35 ± 0.24^{e}	0.40	1.4
day #3	28.9	$1.84 \pm 0.21^{a,b}$	0.53	1.7
day #4	24.8	$2.21 \pm 0.65^{a,b}$	0.55	1.9
day #5	5.1	1.17 ± 0.25^{f}	0.06	0
day #6	4.8	1.04 ± 0.41^{f}	0.05	0
SH[5]				
day #2	64.8	$2.54 \pm 0.21^{b,c}$	1.64	4.8
day #3	61.3	2.63 ± 0.30^{c}	1.61	8.1
day #4	63.7	2.88 ± 0.31^{c}	1.83	9.6
day #5	46.3	$2.34 \pm 0.16^{b,c}$	1.08	5.2
day #6	43.7	$2.46 \pm 0.44^{b,c}$	1.07	5.9
DCR[6]				
day #2	52.9	1.93 ± 0.26^{a}	1.02	6.3
day #3	52.6	$2.21 \pm 0.32^{a,b}$	1.16	6.4
day #4	54.7	2.63 ± 0.24^{c}	1.43	4.4
day #5	25.7	$2.31 \pm 0.47^{a,b}$	0.59	1.5
day #6	27.0	$2.26 \pm 0.63^{a,b}$	0.62	2.1
AE[7]				
day #2	41.0	1.46 ± 0.19^{e}	0.60	2.3
day #3	33.4	$1.55 \pm 0.36^{a,b}$	0.53	2.0
day #4	46.3	$2.43 \pm 0.27^{b,c}$	1.13	3.6
day #5	27.5	1.85 ± 0.56^{a}	1.51	2.2
day #6	22.9	$2.79 \pm 1.09^{c,d}$	0.64	0.8

Means followed by same letters are not significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, the explants were maintained under conditions as follows:
age of cotyledon + 10μM BA $\xrightarrow{\text{7 days}}$ 10μM K + 10μM BA $\xrightarrow{\text{7 days}}$ ½ salt concentration of each nutrient medium.

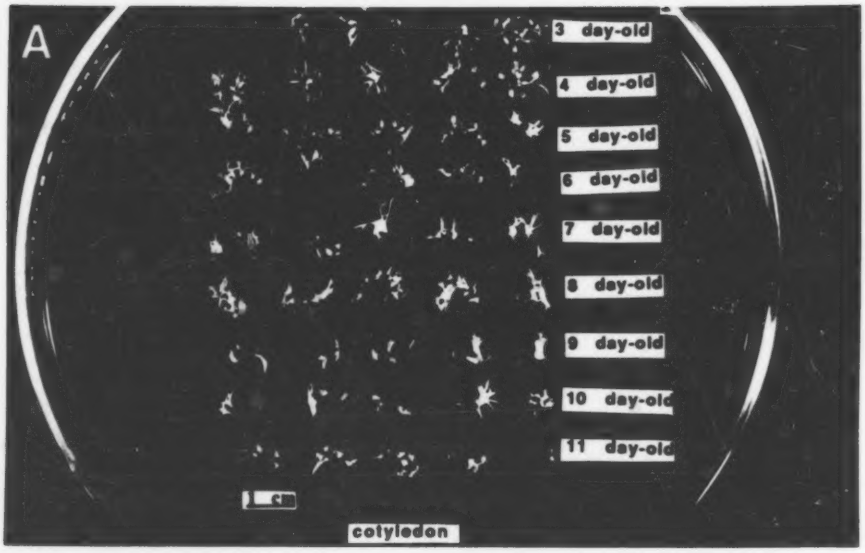
[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation done after 8 weeks in culture. [2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications.

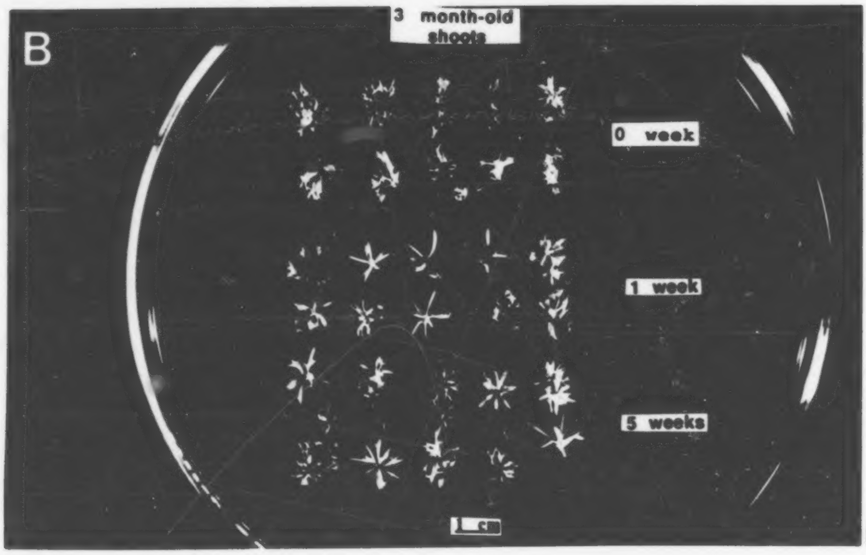
[3] MCM (Bornman, 1983); [4] MS (Murashige and Skoog, 1962); [5] SH (Shenk and Hildebrandt, 1972); [6] DCR (Gupta and Durzan, 1985); [7] AE (von Arnold and Eriksson, 1981).

Figure 1.A. Effect of age of cotyledonary explants (from 3 days to 11 days) of *A. amabilis* on their bud forming capacity (BFC) and shoot elongation capacity (SEC) indexes. The explants were cultivated for the first week in a SH medium supplemented with 10μM of BA and then transferred to equimolar concentrations of BA and Zeatin for the second week maintained at the same level of the first week.

Figure 1.B. General trend of 3-day-old cotyledonary explant responses after the submission to different time of exposure under cold temperature (2° C) and high level of moisture content. The cotyledons were stratified for zero, one and five weeks and cultivated under the same hormonal and nutritive conditions as depicted in figure 1A.

Note: the explants were 6-week-old in Fig 1A, and 14-week-old in 1B.


A
3 day-old
4 day-old
5 day-old
6 day-old
7 day-old
8 day-old
9 day-old
10 day-old
11 day-old
1 cm
cotyledon


B
3 month-old shoots
0 week
1 week
5 weeks
1 cm

cotyledons were cultivated under shoot formation (SF) medium. After each stratification period, the explants were plated horizontally on full-strength SH salts, keeping the same conditions outlined earlier.

Table 4 shows the final results of these treatments, where the longest cooling treatment proved beneficial for bud induction and shoot elongation (see fig. 1B, p. 58). This treatment enhanced *de novo* organ formation both qualitatively and quantitatively. Adventitious bud formation in cotyledonary explants was augmented mainly due to an increase in the average number of buds per cotyledon (3.48 against 2.73 for the second best treatment) and in the percentage of cotyledons responding to cytokinins. Also, both BFC and SEC indexes for 5 weeks of stratification (2.21 and 15.5 respectively) increased slightly when compared to zero and one week of stratification (table 4). This represents an increase of 13% in performance over the second best treatment. The elongated shoots coming from cotyledons stratified for 5 weeks were green, healthy, and showed no signs of vitrification.

Another experiment was carried out to compare time of stratification and embryo vigor after their excision from the seed coat and germination *in vitro* for a period of two weeks. Stratification in this case was performed for 0, 1, 2, 3 and 6 weeks. Table 5 and fig. 2 show the results for this experiment. Besides color, the main distinction between mature (green), ripening (yellowish color) and immature (whitish color) embryos was that experiments done previously showed that when all these kinds of embryos were submitted to the same bud induction treatment *in vitro*, they gave different performances. Green and yellowish embryos gave better response than whitish embryos. Usually the latter maintained their color for a longer time and frequently failed to respond to *in vitro* bud induction.

Another interesting result detected from the stratification treatments presented in Table 5 was that when both percentages of green and yellow stages of embryos were

Table 4. Explant responses of *A. amabilis* to cool temperature (stratification) on their bud forming capacity and shoot elongation capacity indexes, percentage response to hormones, number of shoots elongating and total number of buds per treatment.

Stratification (*)	% of response	No. of buds per cotyl. $X \pm SE$	range	BFC[1] index	Number of buds	Shoots elongating	SEC[2]
0 week	53.8	2.39 ± 0.32^a	1-11	1.51 ± 0.16^a	334	262	13.2
1 week	57.9	2.67 ± 0.30^a	1-7	1.34 ± 0.17^a	297	234	13.6
5 weeks	63.6	3.48 ± 0.14^b	1-17	2.21 ± 0.14^b	495	343	15.5

Means followed by different letters are significantly different at $p \leq 0.05$ by Duncan Multiple Range Test.

(*) For all treatments, 3-day old cotyledonary explants were maintained under conditions as follows: SH[3] 10μM BA $\xrightarrow{\text{7 days}}$ SH 10μM zeatin + 10μM BA $\xrightarrow{\text{7 days}}$ ½ SH[4]

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation performed after 10 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation performed after 18 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

Table 5. Effect of different times of cooling treatments on embryo vigor of *A. amabilis*.

Period of stratification	Number of seeds (A)	(A) %	Greenish embryos (B)	(B) %	Yellowish embryos (C)	(C) %	Whitish embryos (D)	(D) %
0 week	186	100	47	25.3	39	21.0	100	53.7
1 week	182	100	58	31.9	53	29.1	71	39.0
1 week*	64	100	32	50.0	12	18.8	20	31.2
2 weeks	191	100	83	43.5	69	36.1	39	20.4
3 weeks	211	100	70	33.2	106	50.2	35	16.6
6 weeks	182	100	155	85.2	24	13.2	3	1.6

Chi-square goodness of fit for combination of mature and ripening (greenish and yellowish) against immature (whitish) has been found significant at $p \leq 0.001$. The sterilized seeds were submitted to zero (control), one, two, three and six weeks of stratification and then dissected out from the seed coat and the embryos were germinated *in vitro* for two weeks.

Note: evaluation of the experiment was performed after two weeks, and for all the treatments the explants were germinated just in 1% sucrose and 0.7% Noble agar®.

(*) Only the best seeds were selected after stratification.

added they showed an increase with time of stratification, reaching the highest value at the longest cooling treatment. This trend reinforces the numbers indicated in Table 4, substantiating the hypothesis that stratification treatments increase the embryos' vigor and promote bud formation *in vitro* (Fig 2.C).

2.3. Selection of the Optimum Medium

The influence of nutrient medium and their concentrations are critical factors for promoting a high yield of adventitious buds (von Arnold and Eriksson, 1981; Patel and Thorpe 1984). To determine the optimum nutrient medium, six different basal media in full- and half-strength were tested (MS, MCM, DCR, QP, AE, and SH), and of these SH and MCM full-strength salts formulation produced the best responses. However, the buds were more vigorous on SH medium, and this salt formulation was used as the best basal medium for bud induction. Using this medium, the 3-day-old cotyledonary explants produced the best average number of buds per cotyledons (3.18 ± 0.37), total number of adventitious buds (234) and shoots (153) elongating after 18 weeks (Table 6). From these elongating shoots, 52 were higher than 5 mm and subsequently reached the stage for rooting within the first 18 weeks in culture. Also the SEC index was the highest (11.3) of all the salt concentrations compared. These elongated shoots were vigorous, without presence of vitrification or dormancy and displayed a dark green color. The second best nutrient medium was MCM, and the average numbers of buds obtained were very close to SH medium, but the latter ranked better in an overall view; therefore this salt formulation was selected as the best basal medium for bud induction. Comparison of half-strength salt formulation gave contrasting results, but they were inferior to the best results for full-strength concentrations (see table 6).

Bud growth or development on the explants was achieved by subculturing the explants onto a half-strength SH salts devoid of any cytokinins. By this time the explant had been cultivated in presence of combinations of 10μM BA for the first week and

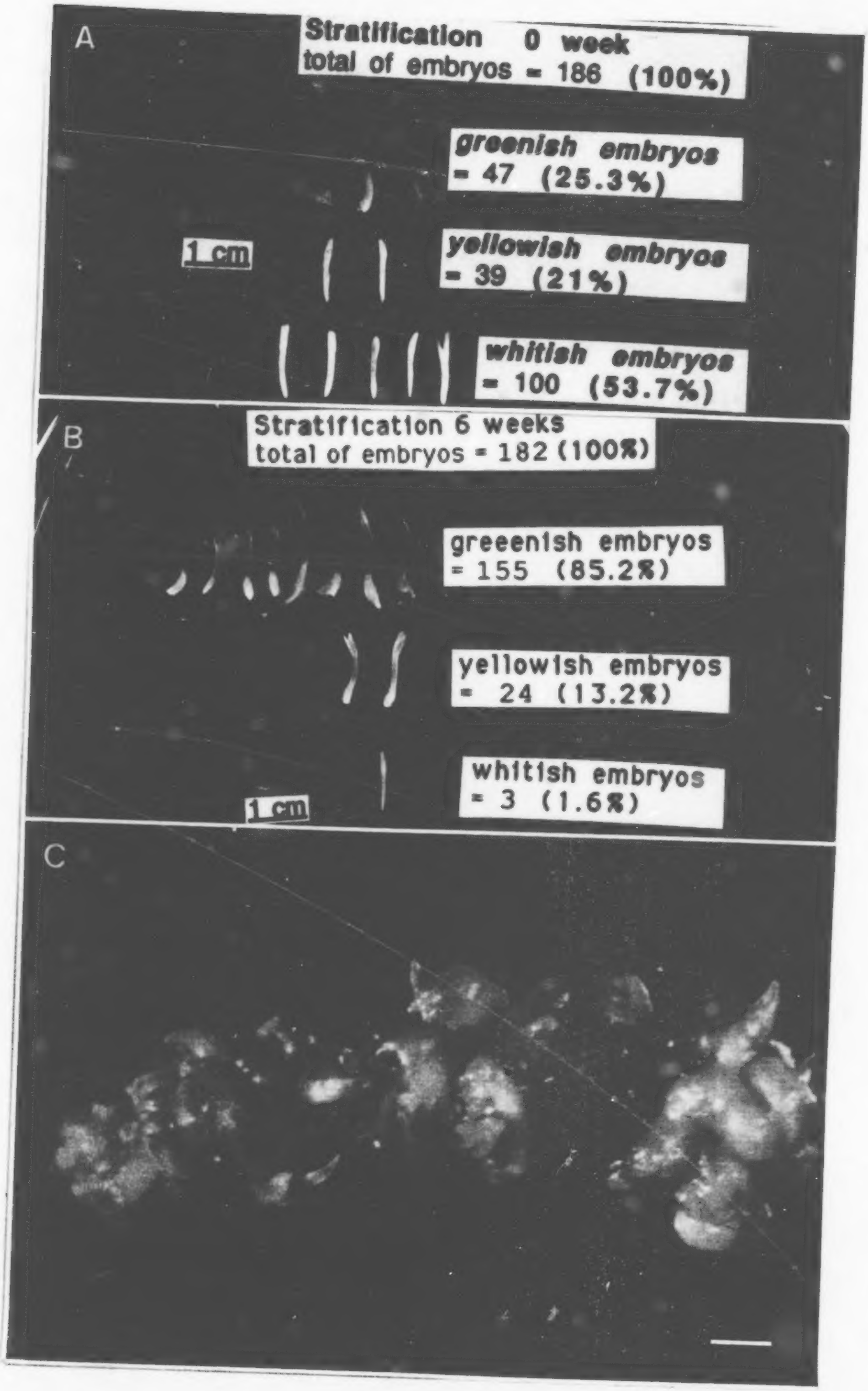
Figure 2. Effect of different times of cooling treatment on embryo vigor of *A. amabilis*. The sterilized seeds were submitted to 0 (control), 1, 2, 3, and 6 weeks of stratification and the seeds were then dissected out and the embryos were germinated *in vitro* in a sucrose-agar medium for 2 weeks.

2.A. Embryos germinated *in vitro* for 2 weeks without previous stratification (control).

2.B. Embryos from seeds stratified for 6 weeks and germinated *in vitro* for 2 weeks.

2.C. Cotyledonary explant coming from seeds stratified for 6 weeks.

Key: bar corresponds to 1.5 mm.


A
Stratification 0 week
total of embryos = 186 (100%)
greenish embryos = 47 (25.3%)
1 cm
yellowish embryos = 39 (21%)
whitish embryos = 100 (53.7%)
B
Stratification 6 weeks
total of embryos = 182 (100%)
greeenish embryos = 155 (85.2%)
yellowish embryos = 24 (13.2%)
whitish embryos = 3 (1.6%)
1 cm
C

equimolar concentration of BA and zeatin at 10μM for the second week. After the transfer to this bud development medium, subculturing was done every two weeks. This approach was necessary, as after this time the explants developed a gradual accumulation of phenolics affecting further development of the buds. The concentration of sucrose, agar and other factors were maintained at the levels of the induction medium.

2.4. Effect of Concentration and Time of Exposure in Cytokinin

The concentration of cytokinin and time of exposure *in vitro* have dramatic effects on the number of adventitious buds, overall shoot quality, and also in the subsequent growth rate of the shoots produced (see Table 7). Until the point at which these experiments were performed, the optimum level of phytohormones that effectively promoted bud induction and shoot elongation was 10μM BA used for the first seven days in culture and equal concentration of BA and kinetin or BA and zeatin for the subsequent seven days, after which the explants were transferred to a medium devoid of cytokinins.

2.5. Effect of Type and Concentration of Phytohormones

The type of cytokinin employed to induce bud formation is a critical component of micropropagation *in vitro,* and each conifer species may differ in their specific requirements (von Arnold, 1982; Rumary and Thorpe, 1984; Abdullah *et al.*, 1987; Saravitz *et al.*, 1991). There are several classes of compounds and several individual compounds which are presently known to have different regulatory effects on growth and development in whole plants (Thorpe, 1980). BA is one of the most prolific in inducing adventitious budding, and thus is the most commonly used cytokinin (Thorpe, 1980). Thus the effects of various cytokinins, alone or in combination with each other and auxins, their concentrations, and length of exposure to 3-day-old cotyledons of *A. amabilis* were examined. Initials experiments showed that BA at a concentration of 10μM for 14 days gave a good response with 3-day-explants (table 14, p.79).

Table 6. Effect of full- and half-strength of salt concentrations on bud induction and shoot elongation of cotyledonary explants of *A. amabilis*.

Nutrient media (*)	Explant response %	No. of buds per cotyledon X ± SE	BFC[1] index	Total number of buds	Shoots ≥ 5 mm	SEC[2] index
MS[3]	34.6	$2.37 \pm 0.21^{a,b}$	0.82 ± 0.07^{a}	90	(47)	1.5
MCM[4]	66.7	$2.86 \pm 0.29^{a,c,d}$	$1.91 \pm 0.12^{e,f}$	211	(146)	10.4
DCR[5]	69.1	$2.25 \pm 0.13^{a,b}$	$1.50 \pm 0.06^{b,d}$	172	(98)	4.2
QP[6]	51.9	$2.36 \pm 0.15^{a,b}$	$1.35 \pm 0.16^{c,d}$	137	(102)	5.0
AE[7]	35.2	$2.64 \pm 0.31^{a,b,c,d}$	0.93 ± 0.04^{a}	103	(59)	2.5
SH[8]	65.4	$3.18 \pm 0.37^{c,d}$	2.07 ± 0.09^{f}	234	(153)	11.3
½ MS	47.1	2.09 ± 0.25^{b}	$1.12 \pm 0.11^{a,c}$	126	(87)	3.1
½ MCM	54.8	$3.09 \pm 0.16^{c,d}$	$1.69 \pm 0.21^{d,e}$	195	(131)	5.8
½ DCR	46.5	2.02 ± 0.21^{b}	0.94 ± 0.07^{a}	106	(72)	2.6
½ QP	53.0	$2.46 \pm 0.22^{a,b,c}$	$1.30 \pm 0.08^{b,c}$	151	(94)	6.4
½ AE	43.3	$2.84 \pm 0.17^{a,c,d}$	$1.23 \pm 0.06^{b,c}$	144	(65)	3.2
½ SH	49.2	$2.77 \pm 0.30^{a,c,d}$	$1.36 \pm 0.10^{b,c}$	194	(108)	4.8

Means followed by the same letters are not significant different at $p \leq 0.05$ using Duncan Multiple Range Test

(*) For all treatments, 3-day old explants were maintained under conditions as follows: nutrient medium + 10μM BA $\xrightarrow{7 \text{ days}}$ nutrient medium 10μM zeatin + 10μM BA $\xrightarrow{7 \text{ days}}$ half-strength nutrient medium

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 10 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 18 weeks in culture.

[3] MS (Murashige and Skoog, 1962); [4] MCM (Bornman, 1983); [5] DCR (Gupta and Durzan, 1985); [6] QP (Quoirin and LePoivre, 1977); [7] AE (von Arnold and Eriksson, 1981), and [8] SH (Shenk and Hildebrandt, 1972). All nutrient media preceded by 1/2 means the origianl medium was reduced to half-strength of major salts concentration.

As mentioned before, the synergistic effect of the cytokinins was apparent since attempts to induce *de novo* organ formation using a single cytokinin had failed. Also, the optimum period of culture in the presence of cytokinins is a very important factor and the optimum for this species was found to be 14 days or very close to this time, based in different quantitative and qualitative factors, such as percentage of cotyledons forming buds (71.8%), average number of buds per cotyledons (4.0±0.51), BFC index (2.87) and SEC index (6.9).

To check out these comparative numbers see Table 7. Shorter time of exposure in these cytokinin levels led to sub-optimal development of the buds. After they reached a certain degree of development the apical shoots stayed dormant or grew slowly. In contrast, while high concentration of cytokinin induced accumulation of phenolics and promoted necrosis on the bases of the explants. Also, in high concentrations of phytohormones, the adventitious buds tended to merge together creating a mass of organized tissue. In addition, exposures longer than fourteen days in cytokinin were not beneficial for bud elongation, since callus formation and vitrification increased. The vitrified adventitious shoots were dark green, and had soft tissue and aberrant needles.

2.5.1. Effect of Combinations of BA and Auxins

When combinations of auxins and BA were tested to compare their capacity to induce bud formation and bud elongation, the overall results basically showed auxins to be extremely ineffective when applied alone (Tables 8,9). Preliminary results have pointed out the cytokinin BA at 10μM as the one participating actively in the *de novo* formation of cotyledonary explant, it was chosen as the optimum type and concentration of cytokinin to be tried in combination with auxins. The interprtation of both tables 8 and 9 shows that NAA was the most efficient auxin promoting bud induction in *A. amabilis*, particularly when applied at the levels 0.1μM and 1.0μM. These levels combined with

Table 7. Effect of N^6-benzyladenine concentrations and times of exposure in induction and elongation of buds on cotyledonary explants of *A. amabilis*.

Days in cytokinin & BA concentration (*)	%Cotyledon forming buds	No. of buds per cotyledon X±SE	BFC[1] index	SEC[2] >5 mm
1 μM BA				
7 days	26.8	1.52±0.53^{a}	0.24±0.07^{a}	-
14 days	49.6	2.4±0.23^{a}	1.20±0.14^{b}	1.4
21 days	44.1	2.8±0.15^{c}	0.96±0.17^{a,b}	0.7
5 μM BA				
7 days	53.9	4.2±0.24^{b,d}	2.26±0.38^{e,d}	-
14 days	68.5	3.93±0.54^{b,d,c}	3.21±0.32^{c}	3.8
21 days	61.2	3.1±0.19^{c,e}	1.89±0.23^{e}	2.9**
10 μM BA				
7 days	76.3	3.5±0.51^{c,d,e}	2.67±0.14^{e,d,c}	-
14 days	71.8	4.0±0.34^{b,d,e}	2.87±0.60^{d,c}	6.9
21 days	54.4	4.6±0.26^{b}	2.50±0.32^{e,d,c}	2.1 **
50 μM BA				
7 days	46.3	1.9±0.27^{a}	0.88±0.17^{a,b}	1.2
14 days	50.0	3.5±0.19^{c,d,e}	1.75±0.29^{b,e}	1.6 **
21 days	69.7	3.8±0.29^{b,c,d,e}	2.65±0.22^{e,d,c}	**

Means followed by the same letter are not significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, 3-day old explants were maintained under conditions as follows:

SH[3] +(1, 5, 10, and 50μM BA) $\xrightarrow{\text{7 days}}$ SH + 10μM Z +(1, 5, 10, and 50μM BA) $\xrightarrow{\text{(0, 7 or 14 days)}}$ 1/2 SH[4]

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 18 weeks in culture.

(**) formation of callus

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concetration.

10μM BA applied for the first or the second 7-day period, produced the best average number of buds per cotyledon and SEC index respectively. In these cases, the results reflected not only the ranking of numbers but also the quality of shoots.

Concentration of 0.1μM NAA produced very green buds and most of the buds turned into shoots and were able to elongate. NAA at 10μM failed to generate buds, instead it promoted a large quantity of callus and necrotic tissue in the explant. Also, it was noticed that at this level, when combined with BA, some 3-day-old segments of hypocotyls (10 mm long) started to promote adventitious roots, but these roots failed to develop subsequently.

Another feature observed was that of elongation in some of those hypocotyls and cotyledon explants present in these treatments. This gradient was more pronounced at higher levels of auxin, particularly 2,4-D, which at its highest level stimulated the hypocotyls and cotyledonary explants to elongate 3 to 4 times their original size.

Also another notable feature was the formation of spontaneous rooting in the cotyledonary explant (Fig. 12.B, p. 115). This feature was most frequently observed while the adventitious shoots were still attached to the original explants, but also it appeared in advanced stages when isolated shoots were elongating. This rooting effect was more pronounced when 1μM NAA and 2,4-D were used during the first week in combination with BA. In these cases circa of 6% and 4% of the adventitious shoots elongating gave spontaneous rooting.

The best overall formulation considering combinations of auxins and BA was 0.1μM NAA applied during the first week together with 10μM BA, and close to this was 1μM NAA added similarly as the former concentration. Under these conditions 60.0% of the cotyledons responded for both treatments, and the average of buds per cotyledons reached 4.08±0.42 and 3.40±0.23 respectively, but the SEC index turned out to be very low, circa 3.4 and 2.2.

Table 8. Effect of single or combinations of α-naphthaleneacetic acid at 0.1, 1, and 10 μM and 10μM of N^6-benzyladenine for the first or second week on bud induction and shoot elongation on cotyledonary explants of *A. amabilis*.

Treatment NAA *	% cotyledons forming buds	No. of buds per cotyledon X ± SE	BFC[1] index	Number of buds	Shoots ≥ 5mm	SEC[2] index
treat.a	60.0	3.74 ± 0.52^{a}	2.45 ± 0.33^{a}	196	(17)	3.4
treat.b	60.7	3.43 ± 0.38^{a}	2.22 ± 0.39^{a}	180	(11)	2.2
treat.c	3.6	3.60 ± 0.23^{a}	0.82 ± 0.07^{b}	11	0	0
treat.d	0	0^{b}	0^{b}	0	0	0
treat.e	0	0^{b}	0^{b}	0	0	0
treat.f	0	0^{b}	0^{b}	0	0	0
treat.g	40.4	$1.60 \pm 0.11^{c,d}$	$0.65 \pm 0.09^{b,c}$	37	(4)	0.5
treat.h	62.0	2.33 ± 0.14^{e}	1.44 ± 0.14^{e}	84	(13)	2.7
treat.i	16.9	2.00 ± 0.12^{a}	$0.34 \pm 0.05^{c,d}$	20	(2)	0.1
treat.j	12.0	1.58 ± 0.17^{a}	0.18 ± 0.02^{d}	11	0	0
treat.k	3.4	1.00 ± 0.34^{a}	0.03 ± 0.01^{d}	2	0	0
treat.l	0	0^{b}	0^{d}	0	0	0

Means followed by the same letters are not significant different at $p \leq 0.05$ using Duncan Multiple Range Test

(*) For all treatments, 3-day old explants were maintained under conditions as follows:
treat. a,b, and c: SH[3] + 10μM BA + (0.1, 1 and 10μM NAA) $\xrightarrow{7\text{ days}}$ SH + 10μM BA $\xrightarrow{7\text{days}}$ 1/2SH[4]
treat. d,e, and f: SH+ (10, 1 and 0.1μM NAA) $\xrightarrow{7\text{ days}}$ SH + 10μM BA $\xrightarrow{7\text{ days}}$ 1/2SH
treat. g,h, and i: SH + 10μM BA $\xrightarrow{7\text{ days}}$ SH + 10μM BA + (0.1, 1 and 10μM NAA) $\xrightarrow{7\text{ days}}$ 1/2SH
treat. j,k, and l: SH + 10μM BA $\xrightarrow{7\text{ days}}$ SH + (10, 1 and 0.1μM NAA) $\xrightarrow{7\text{ days}}$ 1/2SH

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 10 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 16 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

Table 9. Effect of single or combinations of 2,4-dichlorophenoxyacetic acid at 0.1, 1, and 10 μM, and 10μM of N^6-benzyladenine for the first or second week on bud induction and shoot elongation on cotyledonary explants of *A. amabilis*.

Treatment 2,4-D (*)	% cotyledons forming buds	No. of buds per cotyledon X ± SE	BFC[1] index	Number of buds	Shoots ≥ 5mm	SEC[2] index
treat. a	42.7	4.14 ± 0.15^{a}	$1.76 \pm 0.07^{a,b}$	167	(2)	0.3
treat. b	58.4	2.90 ± 0.17^{b}	$1.64 \pm 0.08^{b,c}$	166	(3)	0.6
treat. c	56.4	2.80 ± 0.38^{b}	$1.59 \pm 0.16^{b,c}$	154	(5)	0.9
treat. d	0	0^{c}	0	0	0	0
treat. e	5.0	1.00 ± 0.22^{d}	0.05 ± 0.02^{e}	5	0	0
treat. f	1.0	2.00 ± 0.15^{e}	0.02 ± 0.01^{e}	2	0	0
treat. g	11.1	1.00 ± 0.16^{d}	0.11 ± 0.03^{e}	7	0	0
treat. h	13.1	1.00 ± 0.21^{d}	0.13 ± 0.06^{e}	8	0	0
treat. i	11.4	1.00 ± 0.23^{d}	0.12 ± 0.04^{e}	8	0	0
treat. j	35.6	2.90 ± 0.26^{b}	1.03 ± 0.14^{d}	61	0	0
treat. k	40.0	3.35 ± 0.62^{b}	1.34 ± 0.15^{c}	94	(2)	0.3
treat. l	66.6	2.93 ± 0.29^{b}	1.95 ± 0.19^{a}	135	(4)	0.9

Means followed by the same letters are not significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, 3-day old explants were maintained under conditions as follows:
treat. a,b, and c: SH[3] + 10μM BA + (0.1, 1 and 10μM 2,4-D) $\xrightarrow{\text{7 days}}$ SH + 10μM BA $\xrightarrow{\text{7days}}$ 1/2SH[4]
treat. d,e, and f: SH + (10, 1 and 0.1μM 2,4-D) $\xrightarrow{\text{7 days}}$ SH + 10μM BA $\xrightarrow{\text{7 days}}$ 1/2SH
treat. g,h, and i: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + (10, 1 and 0.1μM 2,4-D) $\xrightarrow{\text{7 days}}$ 1/2SH
treat. j,k, and l: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + (10, 1 and 0.1μM 2,4-D) $\xrightarrow{\text{7 days}}$ 1/2SH

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) + 100. Evaluation was performed after 10 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 16 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

2.5.2. Effect of Combinations of Two Cytokinins

Previous studies have shown that combinations of cytokinins may be more effective in inducing budding than single cytokinins (Thorpe and Biondi, 1984; Thorpe and Hasnain, 1988). Thus, experiments were designed to compare the effect of combinations of two cytokinins in bud induction and bud elongation on 3-day-old cotyledonary explants of *A. amabilis*. Tables 10 and 11 show that kinetin and zeatin at 0.1μM, 1μM and 10μM levels when combined with BA during the second week, were very successful in inducing and elongating adventitious primordia. At these concentrations the best results for organogenesis were achieved. Zeatin and kinetin at the highest levels demonstrated to be mostly effective in stimulating the second stage of organogenesis of *A. amabilis*, while the explant is still under the effect of phytohormones. At this level (10μM) of zeatin or kinetin the highest number of adventitious buds succeeded in elongation at 16 weeks in culture, and reached the rooting stage of micropropagation.

2.5.3. Effect of Combinations of Three Cytokinins

In order to test if there was still a possibility to enhance organogenesis with the addition of a third cytokinin, a new batch of extensive experimentation was devised. Applications of BA and kinetin were combined with a third combination of 0.1μM, 1μM or 10μM levels of zeatin during a second week in combination with BA and K or applied alone for the third week. Zeatin was selected specifically because it had previously proven to be the best for bud induction and elongation coming from the previous experiments. Up to this point any single type and level of phytohormone applied alone or in combination with others did not excede the results obtained with a single application of 10μM of BA as cytokinin for the first seven days in culture. Due to this reason this level and kind of cytokinin was maintained during first week for this experiment. Tables 13 and 14 and Figure 3 show combinations of 10μM BA applied always during first week followed by 0.1, 1.0 or 10μM levels of kinetin applied in combinations with 0.1, 1.0 and 10μM levels

Table 10. Effect of kinetin at 0.1, 1, and 10 μM applied alone or in combination with 10μM of N^6-benzyladenine for first or second week on bud induction and shoot elongation of cotyledonary explants of *A. amabilis*.

Treatment Kinetin (*)	% cotyledons forming buds	No. of buds per cotyledon X ± SE	BFC[1] index	Number of buds	Shoots ≥ 5mm	SEC[2] index
treat. a	59.2	3.40 ± 0.49^a	2.03 ± 0.18^a	210	(4)	0.8
treat. b	47.2	2.89 ± 0.20^b	1.37 ± 0.17^b	122	(3)	0.5
treat. c	17.0	1.79 ± 0.16^c	0.32 ± 0.07^c	30	0	0
treat. d	37.0	2.92 ± 0.32^b	1.03 ± 0.05^d	92	(4)	0.5
treat. e	57.7	3.07 ± 0.13^b	1.76 ± 0.03^a	171	(7)	1.3
treat. f	41.7	2.33 ± 0.38^d	0.97 ± 0.24^d	70	(11)	1.5
treat. g	31.6	$2.21 \pm 0.17^{c,d}$	0.70 ± 0.03^e	40	(3)	0.3
treat. h	14.5	1.30 ± 0.11^e	0.13 ± 0.04^c	13	0	0
treat. i	14.9	1.18 ± 0.34^e	0.14 ± 0.05^c	12	0	0
treat. j	50.0	3.52 ± 0.31^a	1.76 ± 0.04^a	109	(35)	6.0
treat. k	58.6	4.56 ± 0.39^f	2.67 ± 0.29^f	155	(21)	4.1
treat. l	72.3	3.83 ± 0.28^a	2.77 ± 0.26^f	180	(15)	3.6

Means followed by different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, 3-day old explants after submitted to 5 weeks of stratification were cultured under conditions as follows:
treat. a,b, and c: SH[3] + (10, 1 and 0.1μM K) $\xrightarrow{\text{7 days}}$ SH + 10μM BA $\xrightarrow{\text{7 days}}$ 1/2SH[4]
treat. d,e, and f: SH + 10μM BA + (10, 1 and 0.1μM K) $\xrightarrow{\text{7 days}}$ SH + 10μM BA $\xrightarrow{\text{7days}}$ 1/2SH
treat. g,h, and i: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + (10, 1 and 0.1μM K) $\xrightarrow{\text{7 days}}$ 1/2SH
treat. j,k, and l: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + (10, 1 and 0.1μM K) $\xrightarrow{\text{7 days}}$ 1/2SH

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 16 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

Table 11. Effect of zeatin at 0.1, 1, and 10μM, applied alone or in combination with 10μM of N^6-benzyladenine for first or second week on bud induction and shoot elongation on cotyledonary explants of *A. amabilis*.

Treatment Zeatin (*)	Explants forming buds (%)	No. of buds per cotyledon X ± SE	BFC[1] index	Number of buds	Shoots (≥ 5mm)	SEC[2] index
treat. a	53.4	$3.02 \pm 0.33^{a,c,d}$	2.15 ± 0.23^{a}	203	(24)	4.3
treat. b	20.2	$2.77 \pm 0.25^{a,b,c}$	$0.59 \pm 0.11^{b,c}$	47	0	0
treat. c	18.6	1.91 ± 0.22^{b}	0.35 ± 0.07^{c}	39	0	0
treat. d	49.7	$3.53 \pm 0.18^{c,d,e}$	1.76 ± 0.07^{a}	194	(11)	1.8
treat. e	54.9	2.06 ± 0.20^{b}	1.13 ± 0.09^{d}	126	(8)	1.4
treat. f	45.8	$2.28 \pm 0.45^{a,b}$	1.04 ± 0.10^{d}	116	(9)	1.4
treat. g	44.6	$2.37 \pm 0.14^{a,b}$	1.15 ± 0.10^{d}	57	(4)	0.6
treat. h	24.1	1.92 ± 0.23^{b}	1.33 ± 0.23^{e}	36	(1)	0.0
treat. i	19.9	2.08 ± 0.30^{b}	1.89 ± 0.28^{a}	42	(1)	0.0
treat. j	64.9	4.01 ± 0.39^{e}	3.01 ± 0.22^{f}	217	(41)	8.9
treat. k	51.5	$3.86 \pm 0.30^{d,e}$	2.06 ± 0.26^{a}	135	(29)	4.9
treat. l	62.3	$3.13 \pm 0.55^{a,c,d}$	2.38 ± 0.37^{a}	160	(33)	6.8

Means followed by the different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, 3-day old explants after submitted to 5 weeks of stratification were maintained under conditions as follows:

treat. a,b, and c: $SH^3 + (10, 1 \text{ and } 0.1\mu M\ Z) \xrightarrow{\text{7 days}} SH + 10\mu M\ BA \xrightarrow{\text{7 days}} 1/2SH^4$

treat. d,e, and f: $SH + 10\mu M\ BA + (10, 1 \text{ and } 0.1\mu M\ Z) \xrightarrow{\text{7 days}} SH + 10\mu M\ BA \xrightarrow{\text{7days}} 1/2SH$

treat. g,h, and i: $SH + 10\mu M\ BA \xrightarrow{\text{7 days}} SH + (10, 1 \text{ and } 0.1\mu M\ Z) \xrightarrow{\text{7 days}} 1/2SH$

treat. j,k, and l: $SH + 10\mu M\ BA \xrightarrow{\text{7 days}} SH + 10\mu M\ BA + (10, 1 \text{ and } 0.1\mu M\ Z) \xrightarrow{\text{7 days}} 1/2SH$

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 16 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

of zeatin during second or third week as a single cytokinin. A close look at Tables 12, 13, and 14 reveals that with the exception of the lowest level of zeatin, an extended time under cytokinins did not promote organogenesis and had an adverse effect on cotyledons responding to bud induction. Also zeatin applied at any one of these levels did improve the *de novo* organ formation in cotyledonary explants, but did not increase the shoot elogation index higher than the ones obtained with just two cytokinins. By examining the available data, the overall interpretation is that combinations of lower levels of kinetin and zeatin did not have positive effect on bud formation. An interesting data to compare is that equimolar concentrations of 1μM for K and Z had almost similar effects as equimolar concentrations of 10μM of both cytokinins (compare treatment F of Table 11 with treatment E of Table 12) when the level of BA is maintained at 10μM. This could be an indicator that BA has a quite unique role in *de novo* organ formation of *A. amabilis* when used during the first week in culture.

2.6. Effect of Vitamins

Plant tissue culture media in general require members of the water-soluble or B-group of vitamins, such as thiamine, nicotinic acid and pyridoxine (Dougall, 1972; Thorpe and Patel, 1984). In many cases, these vitamins are added to insure that a deficiency does not occur rather than on the basis of demonstrated need. However, since the autotrophic potential of the target cells is latent, in some cases vitamins could be critical for bud induction and development, and shoot elongation. In order to test if vitamin levels were critical for induction and development of buds, as well as shoot elongation in this species, an experiment was performed employing SH vitamins (Schenk and Hildebrandt, 1972) and the results are depicted in Table 15. Although one (1×) and two (2×) normal doses of vitamins promoted the two highest average number of buds per cotyledon (3.37± 0.63 and 2.79± 0.32 respectively), the supplementation of three (3×) times the normal dose of SH vitamins resulted in the highest number of elongating shoots (108) and SEC

Table 12. Effect of combinations of three cytokinins (N^6-benzyladenine and kinetin at 10μM and zeatin at 0.1, 1.0 and 10μM) on bud induction and shoot elongation on cotyledonary explants of *A. amabilis*.

Treatment Z, K and BA (*)	Explants forming buds %	No. of buds per cotyledon X± SE	BFC[1] index	Number of buds	Shoots (≥ 5mm)	SEC[2] index
treat. A	42.1	3.28 ± 0.14^a	1.07 ± 0.11^a	145	(112)	4.2
treat. B	25.4	1.84 ± 0.13^b	0.47 ± 0.05^b	49	(17)	0.5
treat. C	32.7	3.23 ± 0.31^a	1.06 ± 0.13^a	88	(81)	2.3
treat. D	42.8	3.54 ± 0.25^a	1.54 ± 0.14^a	170	(94)	4.4
treat. E	53.6	3.20 ± 0.36^a	1.71 ± 0.27^c	216	(130)	2.7
treat. F	54.9	3.06 ± 0.54^a	1.70 ± 0.20^c	221	(132)	5.6
Control	63.8	3.47 ± 0.19^a	2.22 ± 0.14^d	291	(147)	7.8

Means followed by different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, the 3-day old explants were maintained under conditions as follows:

treat. A: SH[3] + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + 10μMK $\xrightarrow{\text{7 days}}$ SH + 0.1μM Z $\xrightarrow{\text{7 days}}$ 1/2SH[4]
treat. B: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + 10μM K $\xrightarrow{\text{7 days}}$ SH + 1μM Z $\xrightarrow{\text{7 days}}$ 1/2SH
treat. C: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + 10μM K $\xrightarrow{\text{7 days}}$ SH + 10μM Z $\xrightarrow{\text{7 days}}$ 1/2SH
treat. D: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + 10μM K + 0.1μM Z $\xrightarrow{\text{7 days}}$ 1/2SH
treat. E: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + 10μM K + 1μM Z $\xrightarrow{\text{7 days}}$ 1/2SH
treat. F: SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + 10μM K + 10μM Z $\xrightarrow{\text{7 days}}$ 1/2SH
Control : SH + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + 10μM K $\xrightarrow{\text{7 days}}$ 1/2SH

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 16 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

Table 13. Effect of combinations of three cytokinins (10μM N^6-benzyladenine, 1.0μM kinetin and zeatin at 0.1, 1.0 and 10μM) on bud induction and shoot elongation on cotyledonary explants of *A. amabilis*.

Treatment Z, K and BA (*)	Explants forming buds %	No. of buds per cotyledon $\bar{X} \pm$ SE	BFC[1] index	Number of buds	Shoots ($\geq$ 5mm)	SEC[2] index
treat. A	35.9	2.87 ± 0.31^{a}	0.86 ± 0.12^{a}	102	(72)	1.6
treat.B	20.7	1.45 ± 0.11^{b}	0.39 ± 0.08^{b}	27	(18)	0.2
treat.C	22.8	1.69 ± 0.07^{b}	0.41 ± 0.03^{b}	33	(31)	1.2
treat.D	64.6	$2.41 \pm 0.27^{a,c}$	1.59 ± 0.28^{c}	164	(87)	3.9
treat.E	61.0	2.32 ± 0.19^{c}	$1.42 \pm 0.07^{c,d}$	142	(80)	5.3
treat.F	53.2	1.76 ± 0.14^{b}	0.94 ± 0.06^{a}	94	(72)	2.7
Control 2	49.1	2.34 ± 0.39^{c}	1.10 ± 0.15^{d}	91	(57)	0.7

Means followed by different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, the 3-day old explants were maintained under conditions as follows:

A) SH[3] + 10μM BA $\underline{\text{7 days}}$ SH + 10μM BA + 1μM K $\underline{\text{7 days}}$ SH + 0.1μM Z $\underline{\text{7 days}}$ 1/2SH[4]
B) SH + 10μM BA $\underline{\text{7 days}}$ SH + 10μM BA + 1μM K $\underline{\text{7 days}}$ SH + 1μM Z $\underline{\text{7 days}}$ 1/2SH
C) SH + 10μM BA $\underline{\text{7 days}}$ SH + 10μM BA + 1μM K $\underline{\text{7 days}}$ SH + 10μM Z $\underline{\text{7 days}}$ 1/2SH
D) SH + 10μM BA $\underline{\text{7 days}}$ SH + 10μM BA + 1μM K + 0.1μM Z $\underline{\text{7 days}}$ 1/2SH
E) SH + 10μM BA $\underline{\text{7 days}}$ SH + 10μM BA + 1μM K + 1μM Z $\underline{\text{7 days}}$ 1/2SH
F) SH + 10μM BA $\underline{\text{7 days}}$ SH + 10μM BA + 1μM K + 10μM Z $\underline{\text{7 days}}$ 1/2SH
Control : SH + 1μM BA $\underline{\text{7 days}}$ SH + 10μM BA + 1μM K $\underline{\text{7 days}}$ 1/2SH

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 16 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

Table 14. Effect of combinations of three cytokinins (10μM N^6-benzyladenine, 0.1μM kinetin and zeatin at 0.1, 1.0 and 10μM) on bud induction and shoot elongation on cotyledonary explants of *A. amabilis*.

Treatment Z, K and BA (*)	Explants forming buds %	No. of buds per cotyledon X± SE	BFC[1] index	Number of buds	Shoots	SEC[2] index
treat. A	37.0	2.43 ± 0.20^{a}	0.87 ± 0.05^{a}	97	(31)	1.1
treat.B	58.7	2.94 ±0.11^{b}	1.75 ± 0.17^{b}	198	(73)	4.7
treat.C	57.3	2.46 ±0.26^{a,b}	1.56 ± 0.33^{b}	158	(19)	1.4
treat.D	68.4	2.24 ±0.21^{a}	1.58 ± 0.25^{b}	175	(41)	2.7
treat.E	61.7	2.23 ±0.36^{a}	1.53 ± 0.11^{b}	156	(82)	4.8
treat.F	61.0	2.40 ±0.30^{a,b}	1.49 ± 0.19^{b}	166	(48)	1.2
Control	35.1	1.46 ± 0.04^{c}	0.49 ± 0.02^{a}	57	(18)	-

Means followed by different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, the 3-day old explants were maintained under conditions as follows:

treat. A) SH[3] +10μM BA $\xrightarrow{7\ days}$ SH+10μM BA+0.1μM K $\xrightarrow{7\ days}$ SH+0.1μM Z $\xrightarrow{7\ days}$ 1/2SH[4]
treat. B) SH+10μM BA $\xrightarrow{7\ days}$ SH+10μM BA+0.1μM K $\xrightarrow{7\ days}$ SH+1μM Z $\xrightarrow{7\ days}$ 1/2SH
treat. C) SH+10μM BA $\xrightarrow{7\ days}$ SH+10μM BA+0.1μM K $\xrightarrow{7\ days}$ SH+10μM Z $\xrightarrow{7\ days}$ 1/2SH
treat. D) SH+10μM BA $\xrightarrow{7\ days}$ SH+10μM BA+0.1μM K+0.1μM Z $\xrightarrow{7\ days}$ 1/2SH
treat. E) SH+10μM BA $\xrightarrow{7\ days}$ SH+10μM BA+0.1μM K+1μM Z $\xrightarrow{7\ days}$ 1/2SH
treat. F) SH+10μM BA $\xrightarrow{7\ days}$ SH+10μM BA+0.1μM K+10μM Z $\xrightarrow{7\ days}$ 1/2SH
control : SH+0.1μM BA $\xrightarrow{7\ days}$ SH+10μM BA+0.1μM K $\xrightarrow{7\ days}$ 1/2SH

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 16 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

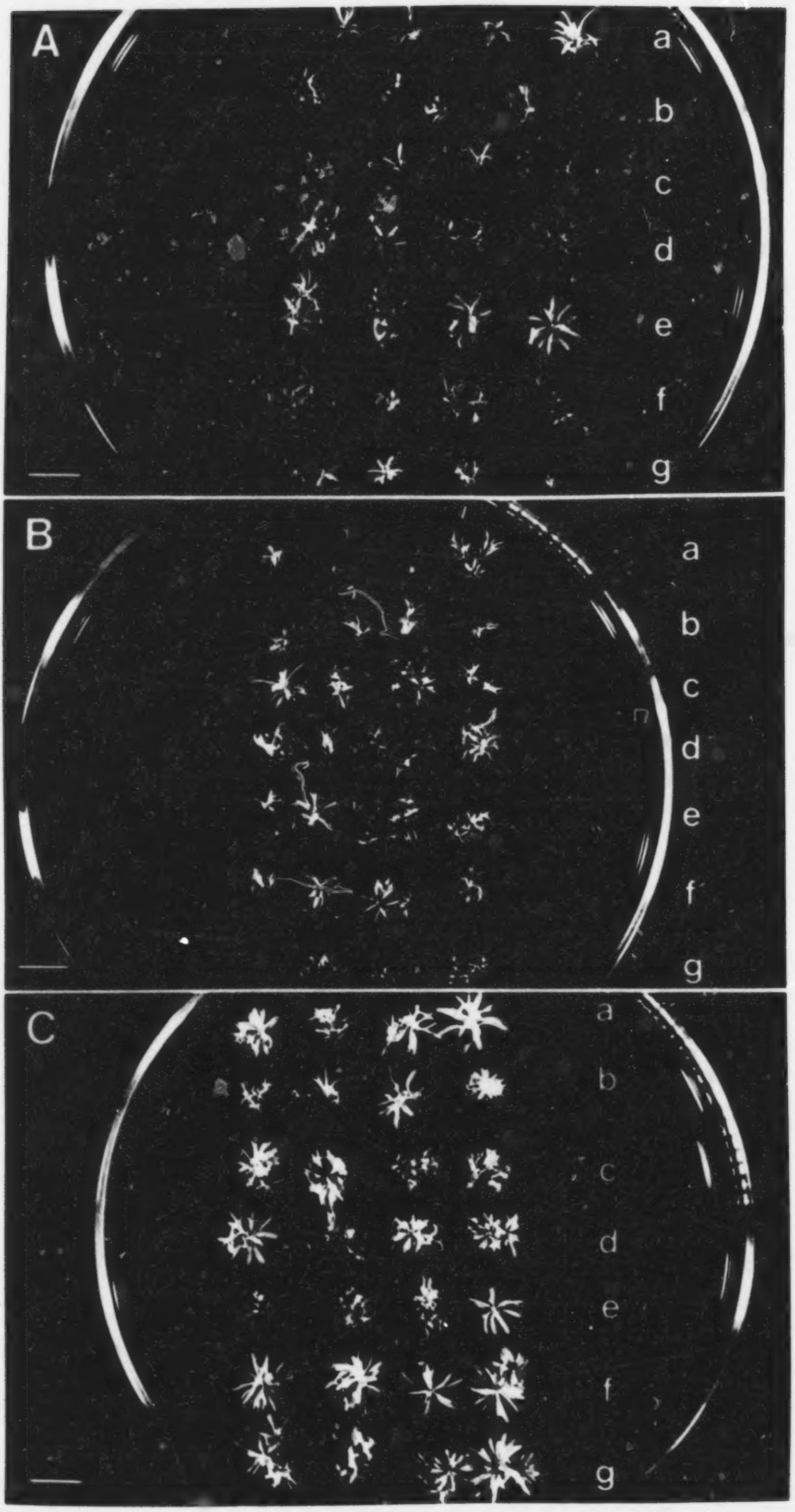
Figure 3. Effect of combinations of three cytokinins (10μM for N^{6}-benzyladenine and kinetin and zeatin at 10, 1, and 0.1 μM) on induction and development of adventitious buds on 3-day old cotyledonary explants of *A. amabilis*. For bud induction treatment all the explants were cultivated in Schenk and Hildebrandt (1972) medium supplemented with BA, kinetin and zeatin, and for bud development the nutrient medium was maintained with the exception that the salt concentration was reduced by half. The evaluation was performed after 18 weeks in culture.

3.A. The 3-day-old cotyledons were cultivated in Schenk and Hildebrandt (1972) medium supplemented with 10μM BA as first for the first seven days and then transferred to similar nutrient medium maintaining BA at 10μM and kinetin at 0.1μM, plus zeatin at (a) 0.1μM; (b) 1μM, and (c) 10μM for an additional week; or zeatin was added alone at (d) 0.1μM; (e) 1μM, and (f) 10μM for the third week. For the control, BA was maintained at 0.1μM during the first week and then transferred to 10μM BA and 0.1μM K during the second week.

3.B. The 3-day old cotyledons were cultivated in Schenk and Hildebrandt (1972) medium supplemented with 10μM BA as first for the first seven days and then transferred to similar nutrient medium maintaining BA at 10μM and kinetin at 1μM, plus zeatin at (a) 0.1μM; (b) 1μM, and (c) 10μM for an additional week; or zeatin was added alone at (d) 0.1μM; (e) 1μM, and (f) 10μM for the third week. For the control, BA was maintained at 1μM as first for the first week and then transferred to 10μM BA and 1μM K for the second week.

3.C. The 3-day old cotyledons were cultivated in Schenk and Hildebrandt (1972) medium supplemented with 10μM BA as first for the first seven days and then transferred to similar nutrient medium with equimolar concentrations of BA and kinetin at 10μM, plus zeatin at (a) 0.1μM; (b) 1μM, and (c) 10μM for an additional week; or zeatin was added alone at (d) 0.1μM; (e) 1μM, and (f) 10μM for the third week. The control as a,b, and but without zeatin.

Key: bars represent 1 cm.


A
a
b
c
d
e
f
g
B
a
b
c
d
e
f
g
C
a
b
c
d
e
f
g

index (10.3) after 16 weeks in culture. These numbers represent 44% and 57% more shoots for rooting than treatments 2× and 1× respectively. This result represents another example pointing out the importance of considering the final product in terms of both qualitative and quantitative parameters. Since treatment 3× resulted in the highest number of vigorous shoots capable of continous development (see fig. 4.A) this level of SH vitamins was used subsequently.

2.7. Effect of Gelling Agents

Gelrite® at 0.2, 0.25, 0.3 and 0.35% was compared with Noble agar® and Difco-Bacto® agar at 1, 0.9, 0.8, 0.7 and 0.6% at the induction stage. The overall picture with these different gelling agents was that only Noble agar® succeeded in producing viable adventitious shoots, and the optimal condition for bud induction was attained at 0.7 %. The remaining agents produced a large quantity of callus and the few elongating buds were generally vitreous and did not survive long in *in vitro* culture.

2.8. Effect of sucrose

Table 16 illustrates the overall results for this experiment, where five different concentrations of sucrose were tested. The different sucrose concentrations were kept constant during the first two stages of each experiment and were only changed when cotyledonary explants were transferred to a half-strength SH salt formulation void of phytohormones, which was intended for bud development. At 8 weeks in culture, the explants were transferred to a half-strength Gupta and Durzan (1983) medium supplemented with 0.05% of activated charcoal, as determined to be optimal for shoot elongation. Concentrations of 30, 60, 90, 120, and 150 mM were tested at induction and development stages and were subsequently maintained at 60 mM with the addition of 0.05% activated charcoal. As Table 16 indicates, the optimum concentration for any stage until shoot elongation was 60 mM sucrose. At this concentration, almost 58% of the

Table 15. Effect of vitamin concentrations on *de novo* organ formation on cotyledonary explants of *A. amabilis*.

Vitamins (*)	% cotyledons forming buds	No. of buds per cotyledon X±SE	BFC[1] index	Number of buds	Shoots elongating		SEC[2] index
					%	total (≥ 5mm)	
1×	59.1	3.37 ± 0.63^{a}	1.93 ± 0.26^{a}	343	13.7	(47)	6.9
2×	49.7	2.79 ± 0.32^{b}	1.34 ± 0.13^{b}	226	26.5	(60)	7.5
3×	38.2	2.74 ± 0.46^{b}	$1.17 \pm 0.36^{c,b}$	222	48.6	(108)	10.3
4×	31.8	2.75 ± 0.23^{b}	$0.77 \pm 0.17^{c,b}$	165	35.2	(58)	5.6
5×	34.7	$2.38 \pm 0.14^{c,b}$	$0.82 \pm 0.08^{c,b}$	149	33.6	(50)	4.3
6×	24.6	2.27 ± 0.31^{c}	0.58 ± 0.15^{c}	113	39.8	(45)	2.8

Means followed by different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) For all treatments, 3-day old explants were maintained under conditions as follows:
SH[3] + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM Z + 10μM BA $\xrightarrow{\text{7 days}}$ 1/2 SH[4]

Each concentration of vitamin was maintained constant for the time of exposure in phytohormones and then changed to standard levels when the explants were transferred to half-strength SH salts concentration.

×= Schenk and Hildebrandt (1972) standard vitamins concentration (see Table 1, p. 40 for composition).

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 18 weeks in culture.

---

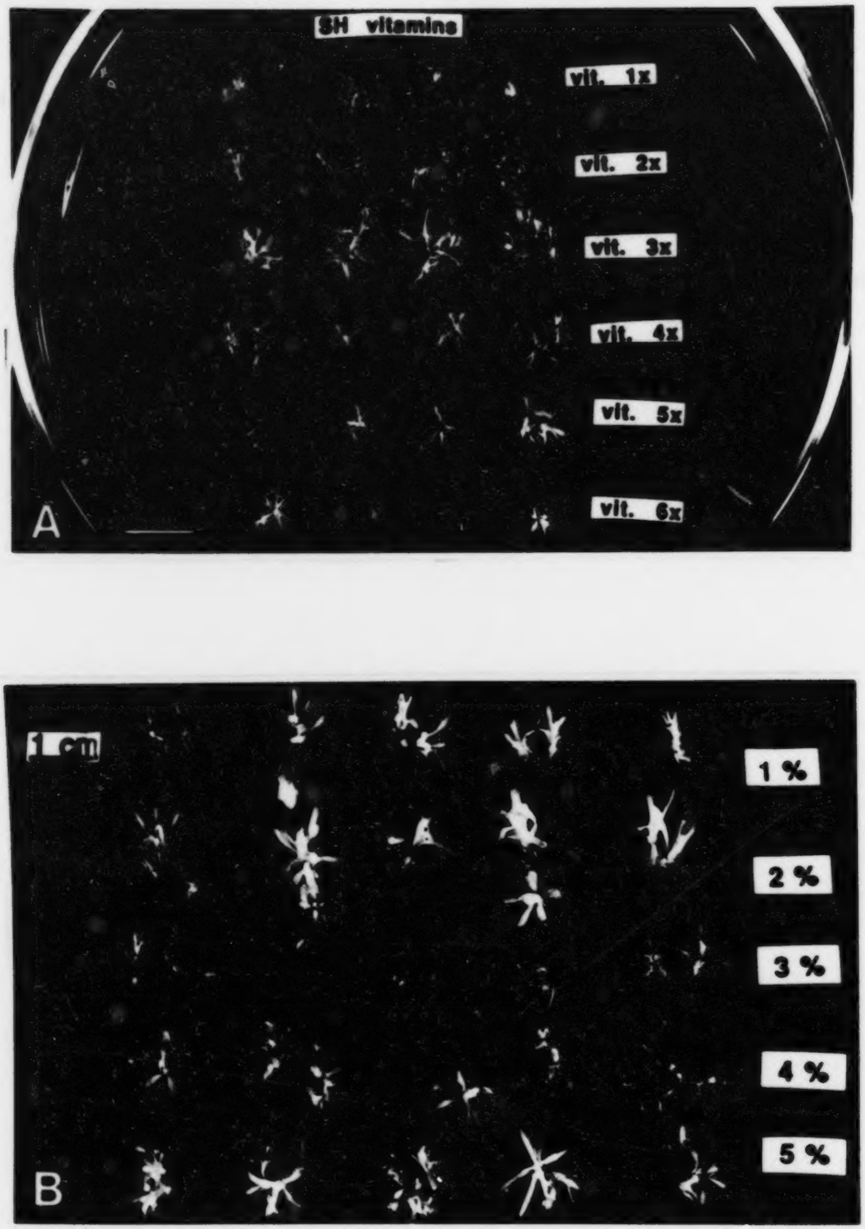
[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concetration.

Figure 4. A. Effect of levels of vitamins on the *de novo* organ formation in 3-day-old cotyledonary explants of *A. amabilis* after 14 weeks in culture. The indication vit. 1×, 2×, 3×, 4×, 5× and 6×, represents times of SH standard vitamins level applied into the nutrient medium containing cytokinins.

B. Effect of levels of sucrose on the *de novo* process of organogenesis in 3-day-old cotyledonary explants of *A. amabilis* after 18 weeks in culture. The indication 1% (30 mM), 2% (60 mM), 3% (90 mM), 4% (120 mM), and 5% (150 mM) represents the individual level of sucrose applied to the nutrient media supplemented with cytokinins.

Key: bar in A represents 1 cm.


SH vitamins
vit. 1x
vit. 2x
vit. 3x
vit. 4x
vit. 5x
vit. 6x
A
1 cm
1 %
2 %
3 %
4 %
5 %
B

cotyledons formed adventitious buds, the average number of buds was 3.17±0.40, the range reached 1 to 21, the BFC index was 1.83 and the SEC index reached the highest value (15.0). In contrast, at 30 mM sucrose, just 38% of the cotyledons formed an average of 2.25 buds, with a range of 1 to 7 buds per cotyledon, and SEC index was minimal (3.62) in comparison with other treatments. Here in this experiment, under every parameter employed to compare data, 60 mM (2%) sucrose was overwhelmingly superior to any other treatment (see fig. 4B, p. 85).

2.9. Effect of "Pulsing" in Epicotyl Explants

Initially, various seedling explants as well as embryonic segments were selected for this experiment. All these explants originated from excised seed embryos germinated in agar-sucrose medium. Also all mineral-salt formulations tried in the previous experiments for bud induction were again selected to be tested for plating the inoculum after pulsing. For gellation, 0.7% Noble agar was always employed.

Studies using epicotyl and hypocotyl segments revealed that the explants survived, remained green, and showed some bud development on MCM and SH salts, thus SH was again the medium of choice. In order to carry out experimentation to determine the possible effect of "pulsing" on induction and development of adventitious bud, all possible embryonic explants were again selected, such as whole embryos, epicotyl and cotyledonary explants. All the potential inocula were immersed in sterilized liquid medium (1mM BA) for 1,2,3 and 4 hours and plated on solid medium for two months. Initially, the age of these explants ranged from 0 to 10-day-old *in vitro* germinated embryos, after which they were then divided into different parts and submitted immediately to "pulsing". Preliminary results indicated the potential of epicotyls and young hypocotyl explants when pulse-treated for 3 hours, and so, both explants were selected for further experiments. These same inocula failed to generate and elongate adventitious shoots, when cultured on solid medium directly.

Table 16. Effect of sucrose concentrations on bud induction and shoot elongation of cotyledonary explants of *A. amabilis*.

Sucrose treatment (*)	% cotyledons forming buds	No. of buds per cotyledon X ± SE	BFC[1] index	Total number of buds	Shoots elongating (> 1cm)	SEC[2] index
30 mM	38.1	2.25 ± 0.10^{a}	0.85 ± 0.11^{a}	311	95	3.62
60 mM	57.7	3.17 ± 0.40^{b}	1.83 ± 0.29^{b}	662	258	15.0
90 mM	58.7	$2.88 \pm 0.28^{c,a,b}$	1.75 ± 0.24^{b}	596	155	10.1
120 mM	64.5	$3.84 \pm 0.22^{c,a,b}$	1.84 ± 0.26^{b}	837	158	11.3
150 mM	56.7	$3.11 \pm 0.37^{b,c}$	1.82 ± 0.31^{b}	615	133	8.4

Means followed by different letters are significant different at $p \leq 0.05$ using Duncans Multiple-Range Test.

(*) For all treatments, 3-day old explants were maintained under conditions as follows:

SH[3] + 10μM BA $\xrightarrow{7\ days}$ SH + 10μM Z + 10μM BA $\xrightarrow{7\ days}$ 1/2 SH[4]

Each concentration of sucrose was maintained constant for the time of exposure in phytohormones and then changed to standard levels (60mM) when the explants were transferred to half-strength SH salts concentration.

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 16 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 18 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concetration.

To carry out further trials with "pulsing", 7-, 14-, 21-, 28- and 35-day-old epicotyl explants (± 5mm), were excised from *in vitro* germinated embryos. The pulse treatments were performed by shaking the epicotyls at 24±1 °C for 3 hours in filter-sterilized 1mM BA solution adjusted to pH 5.5 (Table 17). Epicotyl explants were found to be the optimum for "pulsing". These inoculum were also tested with or without hypocotyl stubs of ± 3 mm, and with trimmed and untrimmed cotyledons' tips (Rumary and Thorpe, 1984).

Epicotyls younger than 21-day-old produced considerable callus and adventitious buds were formed mainly in the cotyledon's suface, but they failed to develop and elongate further. In those explants over 28-day-old the epicotyl tissues continued to develop without any visible sign of adventitious or axillary buds, and the rare cases when they appeared they also failed to elongate into shoots. On the basis of these studies the optimum standardized explants could be selected from 21-day-old seedlings, consisted of untrimmed cotyledons and possessing 3 mm hypocotyl stub (Fig 5). Each 21-day old epicotyl explant has the potential to generate an additional 4-6 axillary shoots with a potential to reach 10 mm high in 12 weeks.

2.10. Effect of "Pulsing" in Cotyledon and Hypocotyl Explants

Newly isolated 3-day-old cotyledons and hypocotyl explants were pulse-treated with BA and transferred to a SH salt formulation supplemented with 60 mM sucrose and 0.7% noble agar. Results are indicated in table 18 and 19. The pulse treatments were carried out by shaking the embryos at 24±1° C for zero (control), one, two and three hours in filter-sterilized 1mM BA solution adjusted to pH 5.0. Control explants were excised from the 3-day embryos and plated directly into the solidified medium containing cytokinins. After the pulse treatment, the explants were transferred to a fresh basal medium and then subcultured each two weeks. Generally speaking, pulse treatment proved

Table 17. Effect of pulsing treatment on bud induction in different ages of epicotyl explants of *A. amabilis*. The inoculum were pulse-treated for 3 hours in 1mM sterilized liquid solution of N^6-benzyladenine.

Age of explant (*)	Explant size (mm)	Explant response (%)	Number of shoots	Buds per epicotyl X ± SE	Range	BFC [1]
0-day old	5	78.4	177	$5.71 \pm 0.99^{a,b}$	1 - 11	$4.45 \pm 0.39^{a,b}$
7-day old	5	83.6	254	6.52 ± 1.08^{b}	2 - 13	5.43 ± 0.47^{b}
14-day old	5	74.4	132	4.57 ± 0.61^{c}	2 - 9	3.38 ± 0.46^{a}
21-day old	5	67.5	191	6.10 ± 1.73^{b}	1 -20	$4.11 \pm 0.57^{a,b}$
28-day old	5	59.3	97	$3.27 \pm 0.40^{d,c}$	1 - 7	1.94 ± 0.29^{c}
35-day old	5	45.8	17	$1.52 \pm 0.30^{e,d}$	1 - 4	0.69 ± 0.16^{c}

Means followed by different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

(*) After the "pulsing" treatment, all explants were maintained under conditions as follows:

$SH^2 + 10\mu M\ BA \xrightarrow{7\ days} SH + 10\mu M\ Z + 10\mu M\ BA \xrightarrow{7\ days} 1/2\ SH^3$

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 10 weeks in culture.

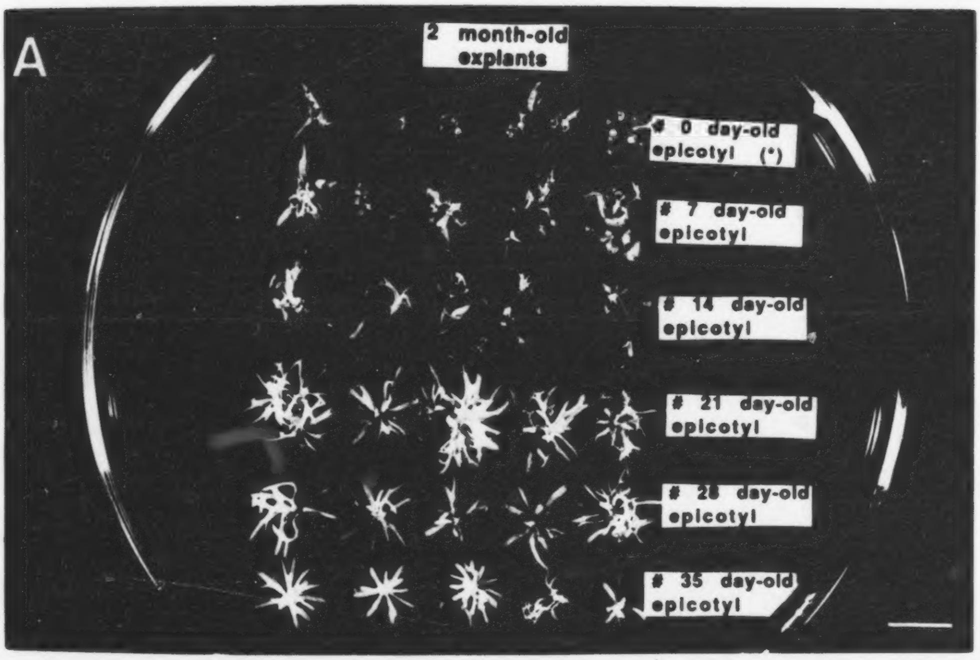
---

[2] Schenk and Hildebrandt (1972) nutrient medium.

[3] Schenk and Hildebrandt (1972) half major salts concentration.

figure 5. Effect of pulse treatment on axyllary bud production in epicotyl explants of *A.amabilis* of different ages. The explants were pulse-treated for 3 hours in high concentration of cytokinin (100μM BA) and then plated vertically in SH nutrient medium.

Key: bar indicates 1 cm.


A
2 month-old explants
# 0 day-old epicotyl (*)
# 7 day-old epicotyl
# 14 day-old epicotyl
# 21 day-old epicotyl
# 28 day-old epicotyl
# 35 day-old epicotyl

to be beneficial for induction and development of adventitious buds in hypocotyl, but not for cotyledonary explants of *A. amabilis* (Fig. 6,7). Preliminary results showed that hypocotyl and epicotyl explants when submitted to shoot-formation medium formed some nodular tissue, but failed to generate elongated shoots. Also, equimolar concentration of 10 μM BA and zeatin during the first and second week in culture, respectively, promoted elongation of adventitious buds on hypocotyls. This appears to be obvious when treatments a,b, and c of Tables 18 and 19 were compared.

Although the average numbers of adventitious buds induced did not appear to have dramatic changes, the quality of elongated shoots increased with the addition of 10 μM zeatin during the second week. Besides the overall qualitative impact, the effect of zeatin can be better visualized when the explants were plated vertically on the surface of the SH nutrient medium. This medium was supplemented with 60mM sucrose, and the explants were subcultured onto a fresh nutrient medium each two weeks.

The best treatment was pulsing hypocotyls for two hours and plating them on solid medium containing 10 μM Z as the subsequent treatment. For this case the SEC index was 14.7, when out of 30 explant segments, 46 buds reached 5 mm high after 14 weeks in culture. Ranking second to this treatment was one hour of pulsing, where the SEC index reached 5.7, accounting for 24 elongated shoots after 14 weeks in culture.

Contrary to the beneficial trend presented for hypocotyls, "pulsing" 3-day old cotyledonary explants in high BA concentration did not improve bud elongation. The high concentration of BA applied for 1, 2 and 3 hours dramatically affected the average number of shoots per cotyledon and also increased the percentage of cotyledons responding to the action of BA. Although there was a substantial increase for BFC index, percentage of response and the number of nodulated formations in the explant, all the buds formed failed to develop and none reached 5 mm high. Even the addition of zeatin as the second treatment did not improve the quality of these adventitious buds visibly. Another feature commonly found as a result of "pulsing" the cotyledons was that the buds had the

Table 18. Effect of "pulsing" on induction and development of adventitious buds on cotyledons and hypocotyls of *A. amabilis*.

Pulsing treatment (*)	Explant size (mm)	Number of buds per explant X±SE	Range	Explant response (%)	BFC[1]	SEC[2]
Cotyledons						
control	3.5 - 4.0	3.14 ± 0.17^{a}	1 - 9	63.6	1.99 ± 0.09^{a}	12.7
1 hour	3.5 - 4.0	3.51 ± 0.24^{a}	1 -12	79.3	$2.78 \pm 0.23^{a,b}$	-
2 hour	3.5 - 4.0	3.73 ± 0.34^{a}	1 -12	61.5	$2.29 \pm 0.40^{a,b}$	-
3 hour	3.5 - 4.0	5.17 ± 0.18^{b}	2 -11	63.4	3.24 ± 0.39^{b}	-
Hypocotyl						
control	10	1.24 ± 0.15^{a}	1 - 3	64.1	0.79 ± 0.17^{a}	6.4
1 hour	10	2.72 ± 0.26^{b}	1 - 5	78.2	2.13 ± 0.28^{b}	2.1
2 hours	10	4.67 ± 0.39^{c}	1 - 9	85.4	3.98 ± 0.14^{c}	3.9
3 hours	10	2.94 ± 0.67^{b}	1 - 5	63.9	1.87 ± 0.20^{b}	-

Means followed by different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

Experiments for cotyledons and hypocotyls were run and interpreted separately.

(*) After the "pulsing" treatment, all 3-day old cotyledons and hypocotyls were maintained under conditions as follows:

control: SH[3] + 10μM BA $\xrightarrow{\text{7 days}}$ SH + 10μM BA + 10μM Z $\xrightarrow{\text{7 days}}$ 1/2 SH[4]
Pulsing for 1 hour in 1mM BA —— SH + 10μM BA $\xrightarrow{\text{14 days}}$ 1/2 SH
Pulsing for 2 hours in 1mM BA —— SH + 10μM BA $\xrightarrow{\text{14 days}}$ 1/2 SH
Pulsing for 3 hours in 1mM BA —— SH + 10μM BA $\xrightarrow{\text{14 days}}$ 1/2 SH

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) ÷ 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 14 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 18 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.

[4] Schenk and Hildebrandt (1972) half major salts concentration.

Table 19. Effect of "pulsing" and plating the explants of *A. amabilis* under different cytokinins in solid media.

Pulsing treatment (*)	Explant size (mm)	Number of buds/explant		Explant response (%)	BFC[1]	SEC[2]
		X±SE	Range			
Cotyledons						
control	3.5 - 4.0	3.40 ± 0.78^{a}	1 - 11	67.2	2.29 ± 0.35^{a}	13.9
1 hour	3.5 - 4.0	$5.64 \pm 0.29^{b,c}$	1 - 14	75.9	4.28 ± 0.37^{b}	-
2 hour	3.5 - 4.0	5.19 ± 0.31^{b}	1 - 16	76.1	3.93 ± 0.41^{b}	0.9
3 hour	3.5 - 4.0	7.26 ± 0.66^{c}	1 - 10	54.8	3.97 ± 0.39^{b}	-
Hypocotyl						
control	10	1.41 ± 0.13^{a}	1 - 4	49.4	0.69 ± 0.16^{a}	3.1*
1 hour	10	2.60 ± 0.20^{b}	1 - 7	58.7	1.52 ± 0.34^{a}	4.7
2 hours	10	4.51 ± 0.38^{c}	1 - 9	73.5	3.31 ± 0.23^{b}	11.2
3 hours	10	4.32 ± 0.22^{c}	1 - 10	74.2	3.21 ± 0.28^{b}	3.8

Means followed by different letters are significant different at $p \leq 0.05$ using Duncan Multiple Range Test.

Experiments for cotyledons and hypocotyls were run and interpreted separately.

(*) After the "pulsing" treatment, all 3-day-old cotyledons and hypocotyls were maintained under conditions as follows:

control for hypocotyls: SH[3] + 10μM BA $\underline{\text{14days}}$ 1/2 SH[4]
control for cotyl.: SH + 10μM BA $\underline{\text{7 days}}$ SH + 10μM BA + 10μM Z $\underline{\text{7 days}}$ 1/2 SH
Pulsing for 1 hour —— SH+10μM BA $\underline{\text{7 days}}$ SH+10μM BA+10μM Z $\underline{\text{7 days}}$ 1/2 SH
Pulsing for 2 hours—— SH+10μM BA $\underline{\text{7 days}}$ SH+10μM BA+10μM Z $\underline{\text{7 days}}$ 1/2 SH
Pulsing for 3 hours—— SH+10μM BA $\underline{\text{7 days}}$ SH+10μM BA+10μM Z $\underline{\text{7 days}}$ 1/2 SH

[1] BFC (bud forming capacity) index = (avg. no. of buds per cotyledon) x (% cotyledons forming buds) + 100. Evaluation was performed after 8 weeks in culture.

[2] SEC (shoot elongation capacity) index = (no. of shoots > 5mm after 14 weeks) x (% cotyledons forming buds) ÷ 100 x number of replications. Evaluation was performed after 18 weeks in culture.

---

[3] Schenk and Hildebrandt (1972) nutrient medium.
[4] Schenk and Hildebrandt (1972) half major salts concentration.

Figure 6. Effect of pulsing treatments on induction and development of adventitious buds in hypocotyl and cotyledonary explants of *A. amabilis*. The explants were pulse-treated in high concentration of BA (1mM) for a period of zero (control), one, two and three hours, and plated horizontally on solid medium containing phytohormones.

A. Pulse-treated 3-day-old cotyledons for a period of zero (a), one hour (b), two hours (c), and three hours (d), and then transferred to solid medium plus single cytokinins as first and as second.

B. Pulse-treated 3-day-old hypocotyls for a period of zero (a), one hour (b), two hours (c), and three hours (d), and then transferred to solid medium plus single cytokinins during first and second week of culture.

Key: bars corresponds to 1 cm.

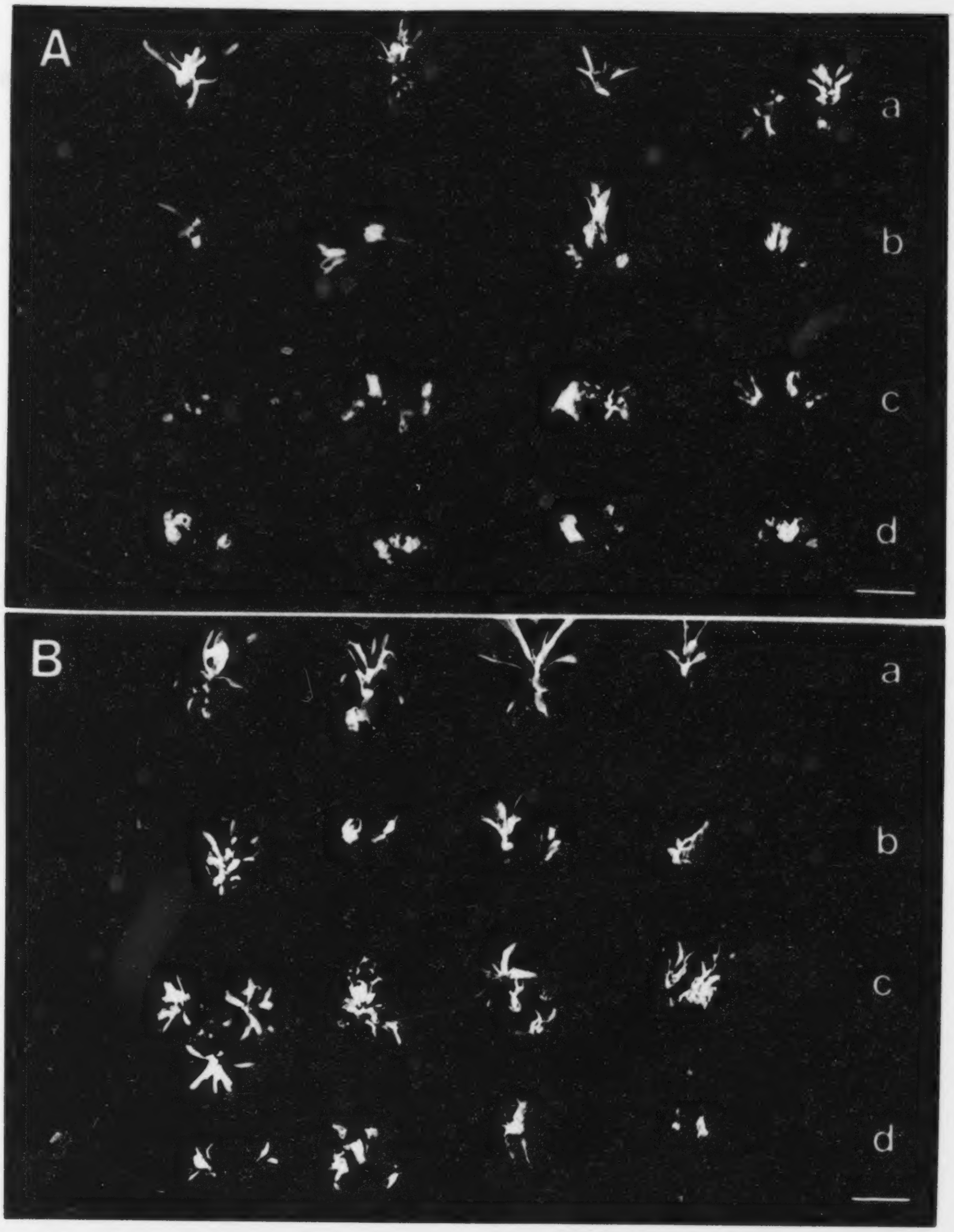
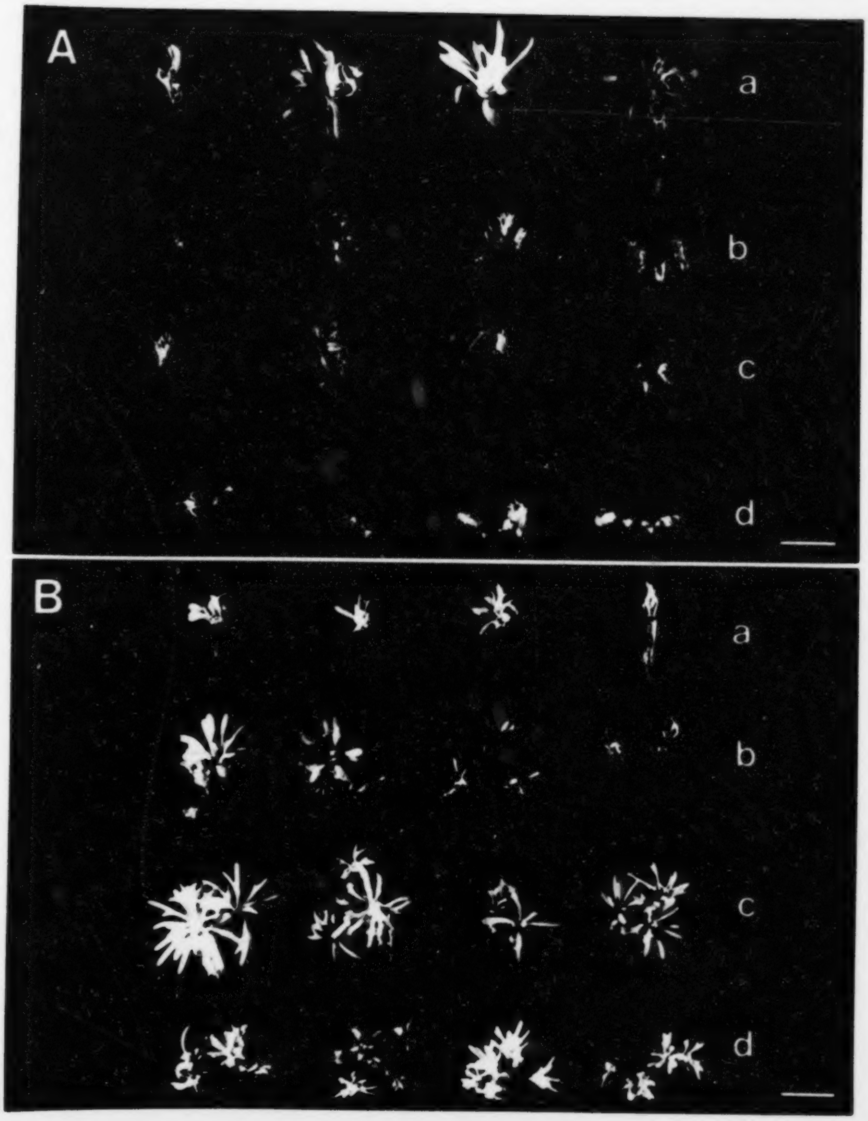

A
a
b
c
d
B
a
b
c
d

Figure 7. Effect of pulsing treatments on induction and development of adventitious buds in hypocotyl and cotyledonary explants of *A. amabilis*. The explants were pulse-treated in high concentration of BA (100μM) for a period of zero (control), one, two and three hours, and plated horizontally on solid medium containing phytohormones.

A. Pulse-treated 3-day-old cotyledons for a period of zero (a), one hour (b), two hours (c), and three hours (d), and then transferred to solid medium plus single cytokinins during first week and combination of cytokinins during the second week in culture.

B. Pulse-treated 3-day old hypocotyls for a period of zero (a), one hour (b), two hours (c), and three hours (d), and then transferred to solid medium plus single cytokinins during first week and combination of cytokinins during the second week in culture.

Key: bars corresponds to 1 cm.


A
a
b
c
d
B
a
b
c
d

tendency to aggregate and grow into a mass of callus. This made it very difficult to properly assess each bud individually.

2.11. Shoot Elongation

The second phase of micropropagation involves multiplication and the development of the nodular tissue into shoots with minimum callus formation. This is usually achieved in a medium lacking phytohormones. Also, amongst many other critical components, several factors have to be tested such as levels of sucrose and major salt formulation and need for low concentrations of phytohormones at later stages of shoot development (Thorpe and Patel, 1984).

For testing the best mineral salt formulation in shoot elongation, 5 mm long, non-vitreous, healthy and green shoots were selected and transferred to storage jars (100 x 80 mm) containing 100 ml of different salts and sucrose concentrations. At first the same original number of salt formulation previously employed to determine the optimum medium for bud induction (SH, MCM, AE, MS, QP and DCR) were tested. Initial results indicated that SH and DCR performed better for shoot elongation and so they were subjected to further investigations.

A 4x3 and 3x3 factorial experiments were performed to determine the best salt and sucrose concentrations for bud elongation employing three levels of sucrose (0, 30, and 60mM) and SH medium at 1, 1/2, 1/3, and 1/4 major salt strength or DCR nutrient medium at 1/2, 1/3, and 1/4 major salt strength. Tables 20 and 21 show the results for both experiments. For these optimization trials all explants were 12-week old, and had an average size of 5 mm. The salt concentrations were solidified with 0.7% of noble agar, with a pH of 5.7 to 5.8 adjusted prior to autoclaving. Once the explants were plated on the medium, they were subcultured every two weeks, and were maintained in a growth chamber where the temperature was kept constant at 24±1°C and 16-h photoperiod provided by Sylvania Gro-lux® F40T12 Gro-WS lamps at a photon fluence rate of about 80 μmol $m^{-2}s^{-1}$.

Sucrose plays a very important role in shoot elongation, where for both combinations of salt formulations (SH and DCR) this source of carbon component had a direct relationship with shoot elongation at a 1% significance level (table 20, 21). From all combinations compared, 1/2 DCR, and 1/4 SH added to 60 mM sucrose provided the best results for average of shoot elongation (11.60 ± 1.05 and 11.22 ± 0.41 mm, respectively) and percentage of shoots elongating (94 and 91%, respectively).

Although no statistical difference was found at the 5% level for the average shoot elongation for the treatment 1/2, 1/3 and 1/4 SH salts as well for 1/2 and 1/4 DCR at 60 mM level of sucrose, the decision in choosing the two above mentioned treatments (1/2 DCR and 1/4 SH) was based on the best average of shoot elongation, percentage of shoots elongating and the best quality of shoots rated in this experiment. Figure 8 depicts the general qualitative trend for both experiments.

2.12. Shoot Multiplication

The number of adventitious shoots produced *in vitro* through embryonic explants of *A. amabilis* was fairly low, and so attempts were made to multiply the primary shoots formed. Figure 8 displays the diversity of adventitious shoots coming from a previous elongated stage. However, it is not uncommon to see adventitious shoots exhibit axillary bud formation when they reach 15 to 20 mm high (figure 9.A,B,C and D, see arrows) but these axillary buds remained in a dormant stage most of time. When these axillary shoots elongated naturally and were excised from the adventitious shoots and cultivated *in vitro* they manifested a strong plagiotropic growth and stayed in this condition even after the rooting stage. Any attempt employing phytohormones (BA, zeatin, kinetin, IAA, NAA at 10, 50 and 100μM) for the purpose of shoot multiplication of *A. amabilis* resulted in a high percentage of callused, stunted, and vitreous shoots (figure 10.A, B, C, and D). Also

Table 20. Effect of a 3x3 factorial for salt concentrations (half-, third- and a quarter-strength) and sucrose (0, 30 and 60 mM) on elongation of 12-week old adventitious shoots of *A. amabilis*.

Sucrose levels x salt concentrations	Initial size of the shoots (mm).	Shoots average after 10 weeks in culture (mm).	% of shoots elongating	shoots general quality (*).
0% sucrose				
1/2 DCR[1]	5.0	5.23 ± 0.11^{a}	0	+
1/3 DCR[2]	5.0	5.46 ± 0.13^{a}	0	+
1/4 DCR[3]	5.0	5.40 ± 0.24^{a}	0	+
1% sucrose				
1/2 DCR	5.0	$7.94 \pm 0.65^{b,c}$	84.2	++
1/3 DCR	5.0	$8.16 \pm 0.72^{b,c}$	86.1	++
1/4 DCR	5.0	11.11 ± 1.03^{c}	89.3	+++
2% sucrose				
1/2 DCR	5.0	11.60 ± 1.05^{c}	91.0	+++
1/3 DCR	5.0	$8.11 \pm 0.50^{b,c}$	87.9	++
1/4 DCR	5.0	10.33 ± 0.73^{c}	90.5	+++

(*) the rating signs means: dead (+); good (++); and best overall shoot quality (+++).
Numbers followed by the same letters means no significant statistic difference at .05 level by Scheffé's test.

---

[1] Gupta and Durzan (1985) half major salts concentration.
[2] Gupta and Durzan (1985) a-third major salts concentration.
[3] Gupta and Durzan (1985) a-quarter major salts concentration.

Table 21. Effect of a 4x3 factorial for salt formulation (full-, half-, third-, and a quarter-strength) and sucrose concentrations (0, 30 and 60 mM) on elongation of 12-week old adventitious shoots of *A. amabilis*.

Sucrose levels X salt concentrations	Initial size of the shoots (mm).	Shoots average after 10 weeks in culture (mm).	% of shoots elongating	Shoots general quality (*).
0% sucrose				
SH[1]	5.0	5.83 ± 0.19^{a}	0	+
1/2 SH[2]	5.0	5.92 ± 0.17^{a}	0	+
1/3 SH[3]	5.0	5.97 ± 0.16^{a}	0	+
1/4 SH[4]	5.0	5.31 ± 0.10^{b}	0	+
1% sucrose				
SH	5.0	7.72 ± 0.40^{a}	88.3	++
1/2 SH	5.0	10.50 ± 0.43^{d}	79.5	+++
1/3 SH	5.0	10.22 ± 0.37^{d}	91.7	+++
1/4 SH	5.0	9.92 ± 0.48^{d}	93.8	+++
2% sucrose				
SH	5.0	8.78 ± 0.43^{c}	85.0	+++
1/2 SH	5.0	10.78 ± 0.34^{d}	92.1	+++
1/3 SH	5.0	11.06 ± 0.47^{d}	89.6	+++
1/4 SH	5.0	11.22 ± 0.41^{d}	94.0	+++

Numbers followed by the same letters means no significant statistic difference at .05 level by Scheffé's test.

(*) the rating signs means: dead (+); good (+ +); and best overall shoot quality (+ + +).

---

[1] Schenk and Hildebrandt (1972) nutrient medium.

[2] Schenk and Hildebrandt (1972) half major salts concentration.

[3] Schenk and Hildebrandt (1972) a third major salts concentration.

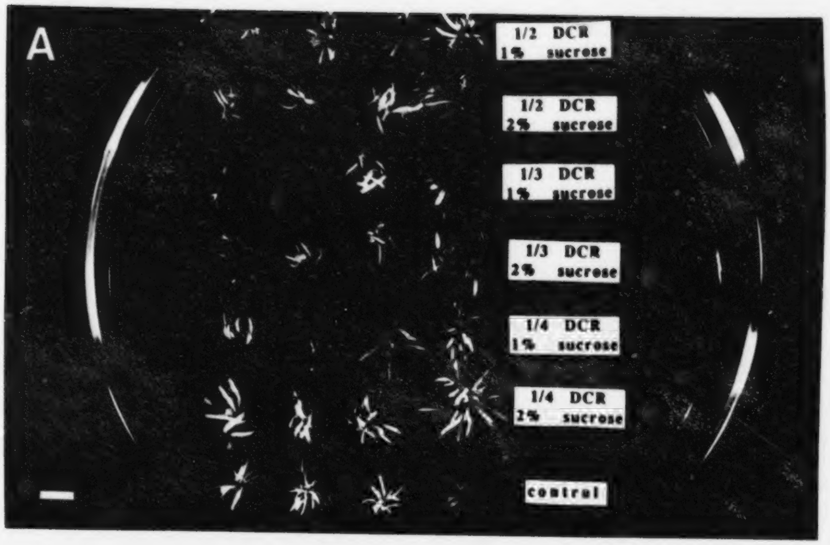
[4] Schenk and Hildebrandt (1972) a quarter major salts concentration.

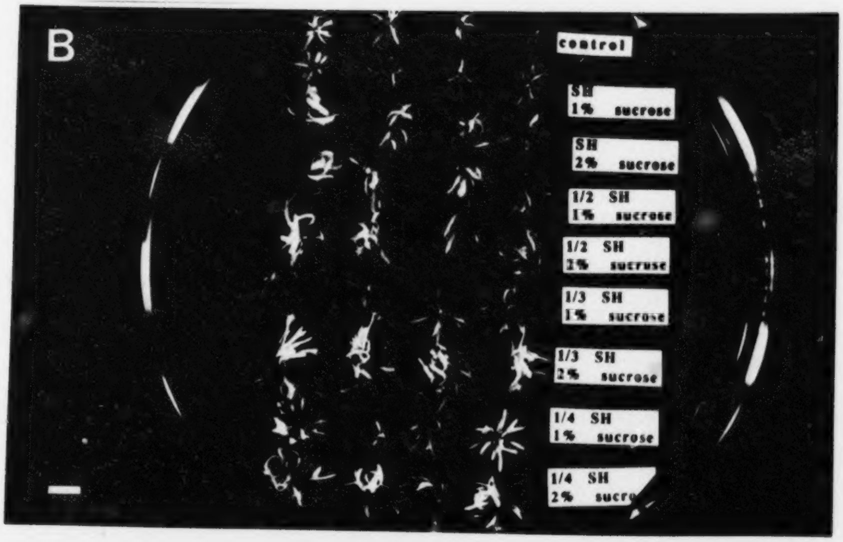
Figure 8: Effect of salt formulation and sucrose concentrations on shoot elongation of cotyledonary explants of *A. amabilis*.

A: Effect of a 3x3 factorial combination of a half-, a third-, and a quarter-strength of DCR's major salts concentration and 0, 30, and 60 mM of sucrose.

B: Effect of a 4x3 factorial combination of full-, a half-, a third-, and a quarter-strength of SH's major salts concentration and 0, 30, and 60 mM of sucrose.

Key: both controls represent treatments lacking sucrose. All shoots in the picture were 16-week-old and the controls presented signals of necrosis. Bars represent 1 cm.


A
1/2 DCR
1% sucrose
1/2 DCR
2% sucrose
1/3 DCR
1% sucrose
1/3 DCR
2% sucrose
1/4 DCR
1% sucrose
1/4 DCR
2% sucrose
control


B
control
SH
1% sucrose
SH
2% sucrose
1/2 SH
1% sucrose
1/2 SH
2% sucrose
1/3 SH
1% sucrose
1/3 SH
2% sucrose
1/4 SH
1% sucrose
1/4 SH
2% sucr

a high percentage of mortality was observed in all these treatments. The addition of any phytohormones to the culture medium for three weeks, did not promote shoot multiplication even at the lowest concentration. Another approach to promote shoot multiplication was the removal of the shoot apex. Apical buds were removed from approximately 20 mm tall shoots and cultivated *in vitro*. After 8 weeks in culture, only a few tiny axillary buds were formed, but most of remained dormant and failed to develop further. The typical feature, shown in the shoots that survived detopping, was a single axillary shoot growing to replace the excised apical formation. The only healthy shoots found were the controls that were not submitted to decapitation of the apical meristem or to hormone treatments. These shoots continued to elongate, were non-vitreous and showed potential for rooting.

2.13. Adventitious Root Formation

The rooting phase is carried out asceptically and employs auxin as adventitious rooting inducer. IBA is generally employed at levels of 0.1-100μM alone or in combination with other auxins or cytokinins either in the medium or as a dip prior to planting (Thorpe and Patel, 1984). Other factors tested are level of sucrose; concentration of mineral salts, length of time in phytohormone and temperature (Thorpe and Patel, 1984). In order to induce adventitious roots in *A. amabilis*, two distinct explants were used: (1) derooted seedlings, also called hypocotyl cuttings, and (2) elongated adventitious shoots. Both explants were approximately 10-20 mm tall. For the former kind of explants the best results appeared to be when"pulsing" in 1mM IBA was performed for 2,3 and 4 hours and then transferred to glass jars containing vermiculite, ready-earth (Terralite®) and sand (1:1.5:1.5) supplemented with 1/4SH and 30mM sucrose. In this case the percentage of rooting after 6 months in rooting medium was 23, 17 and 27% of the total explants treated (Fig. 11A,C). Also, treating with NAA at 1.0, 10 and 20μM promoted callus and adventitious roots. In these cases, the percentage of response was 11, 22 and 15% of the

Figure 9. Prototypes of adventitious shoots of *A. amabilis* at the end of the elongation stage, displaying potential axillary bud formation.

A. Four different shoot prototype representatives of the adventitious shoots formed on cotyledonary explants at 20-week-old. Small arrows show axillary shoots formation and scale indicates 10 mm.

B. An enlargement of a previous adventitious shoot prototype displayed at picture 9.A at the bottom, close to the scale. This shoot presents four axillary buds that could have potential for shoot multiplication. Scale represents 5 mm.

C. A higher magnification of axillary buds being formed in a normal adventitious shoot of *A. amabilis*. These dorment shoots normally appear when the shoots are still elongating. Scale corresponds to 2 mm.

D. A close-up of two healthy and potential would-be axillary shoots for the multiplication stage of *in vitro* mass propagation of *A. amabilis*. Scale corresponds to 1 mm.

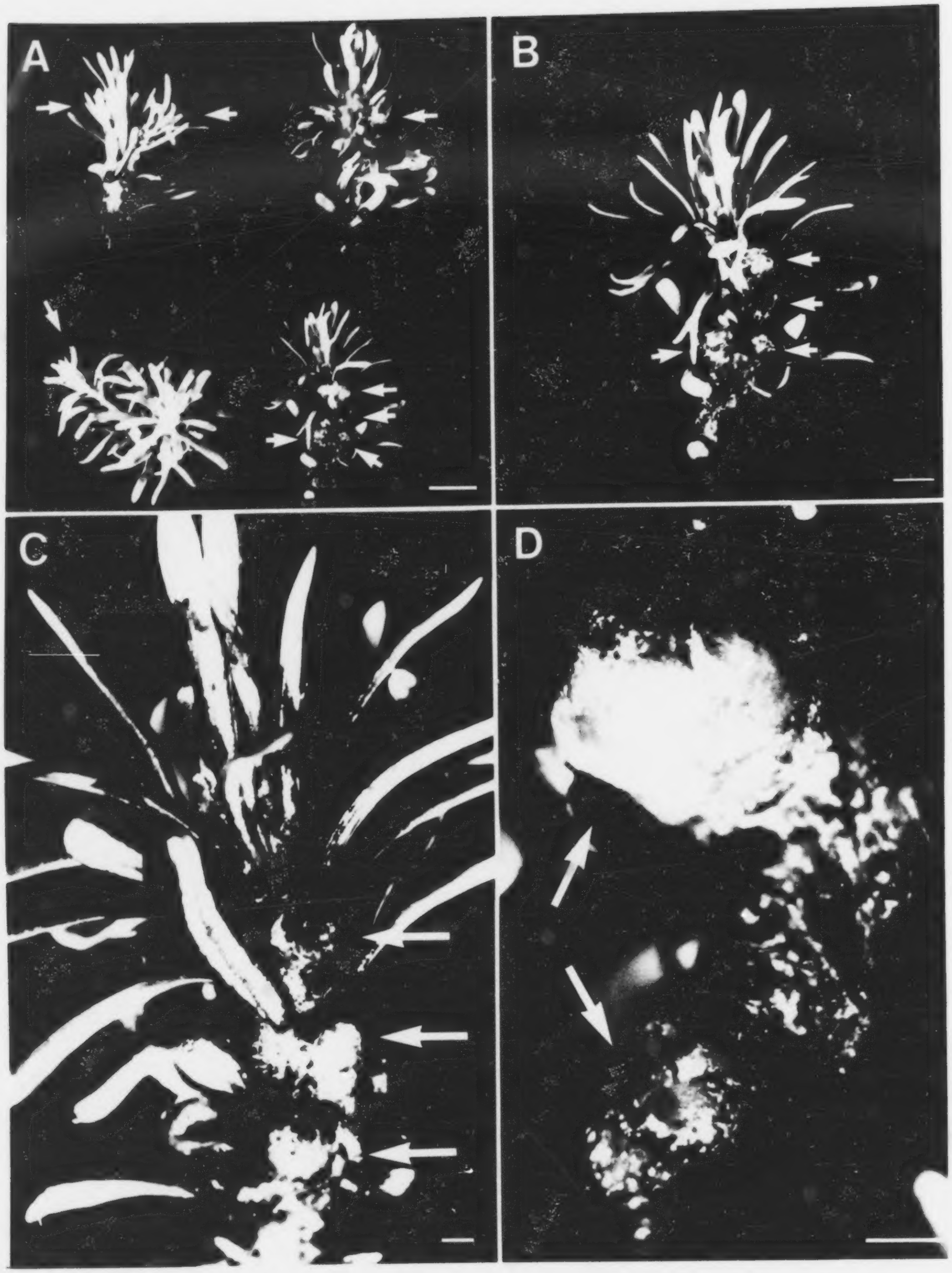

A
B
C
D

Figure 10. Effect of different treatments to induce formation and development of axillary shoots in 20-week-old adventitious shoots of *A. amabilis*.

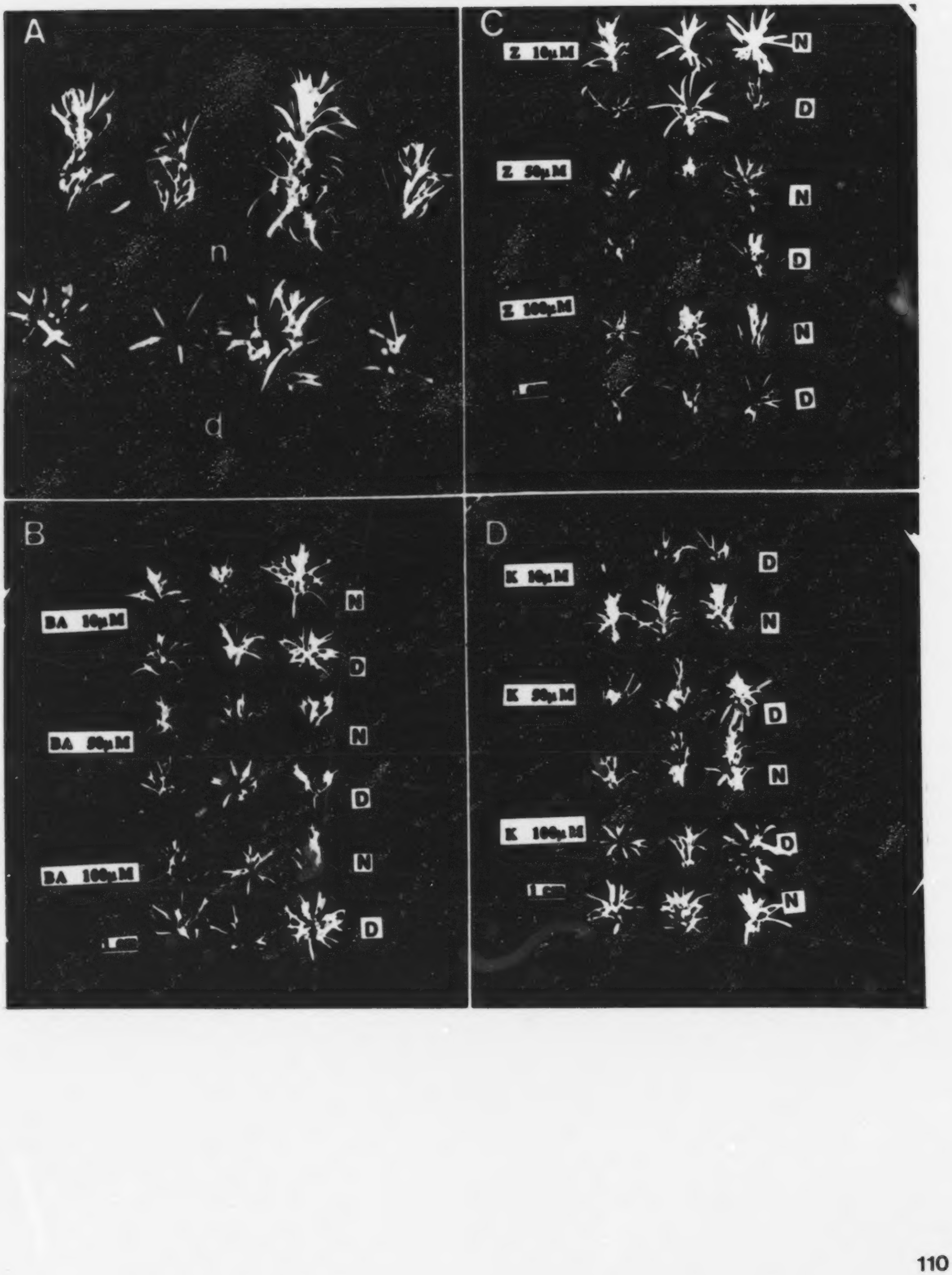
A. picture displaying normal (n) and decapitated (d) controls of 20-week-old adventitious shoots after 10 weeks in half-strength multiplication media.

B. 20-week-old adventitious shoots exposed to BA (10, 50 and 100μM) for 3 weeks, after 7 weeks in culture.

C. idem B, but using zeatin at 10, 50 and 100μM.

D. idem B and C, but employing kinetin at 10, 50 and 100μM.

Key: normal shoots (n) and decapitated shoots (d).


A
n
d
B
BA 10µM
BA 50µM
BA 100µM
N
D
N
D
N
D
C
Z 10µM
Z 50µM
Z 100µM
N
D
N
D
N
D
K 10µM
K 50µM
K 100µM
D
N
D
N
D
N

15% of the total derooted seedlings, but the quality between these treatments was dramatically different (fig. 11B). However, with the exception to 1μM level, the adventitious root primordia produced turned necrotic and only few survived. Rooting of adventitous shoots (fig. 12A) when compared with hypocotyl cutting showed very poor results after 6 months in rooting substrate. Incubation of adventitious shoots with single or combinations of auxins (IAA, 2,4-D, IBA, and NAA) at 1, 10 and 100μM levels as devised in sections 2.4.3 and 2.4.4 of Material & Methods (p.38 and 39) did not promote rooting, instead they produced a mass of callus, abundancy of phenolics and necrotic tissue. Also the shoots turned yellowish after 10 weeks in the soil substrate. The soil substrate was composed of perlite;vermiculite (1:1) and was sterilized in the autoclave prior to use. The only exception was NAA applied at 1 and 10μM when supplemented with SH nutrient medium and 60 mM sucrose. These levels were able to promote very small root primordia (10%) that failed to elongate further (see fig 12C). Pulsing in sterilized solution of 1mM IBA for 3 and 4 hours hours and plating the shoots in a SH medium supplemented with 30 mM sucrose presented the best results for rooting. In these cases, 14 and 17% of the adventitious shoots produced roots, respectively. In both cases, the size of the adventitious roots differed dramatically, (fig. 12C).

There were basic similarities in results in rooting in *A. amabilis* from both kind of explants: (1) formation of coarse roots, (2) frequently just one or two adventitous roots were formed, (3) a swollen region was produced at the base of the shoots, from which a large amount of callus was formed later on. Finally, the most significant of all was that IBA, especially when applied in high concentration (1mM) as pulsing treatment was the most effective rooting inducerr for both type of explants.

The major differences was that adventitious shoots rooted less frequently then hypocotyl cuttings, and the quality of the roots was inferior. Also the survival for the former was very low, since most of the adventitious shoots died within the first or second month under rooting treatments. Most important, the roots generated in hypocotyl cutting

Figure 11. Effect of different rooting treatments for derooted seedlings of *A. amabilis*. After each specific treatment, the explants were plated vertically on soil substrate. This soil substrate consisted of 1:1 peat:vermiculite, and the final evaluation was done after 6 months in culture. The controls were cultivated continuously in soil substrate just containing distilled water.

A. Pulse-treated explants in high concentration of filter-sterilized 1mM IBA solution for 4,3 and 2 hours and then plated on soil substrate.

B. Hypocotyl explants cultivated for 6 weeks in a solidified medium containing NAA at 20, 10 and 1μM, and then transferred to a soil substrate.

C. An overview of the best rooting treatments when compared to normal seedlings germinated *in vitro*.

Key: bars corresponds to 1 cm.

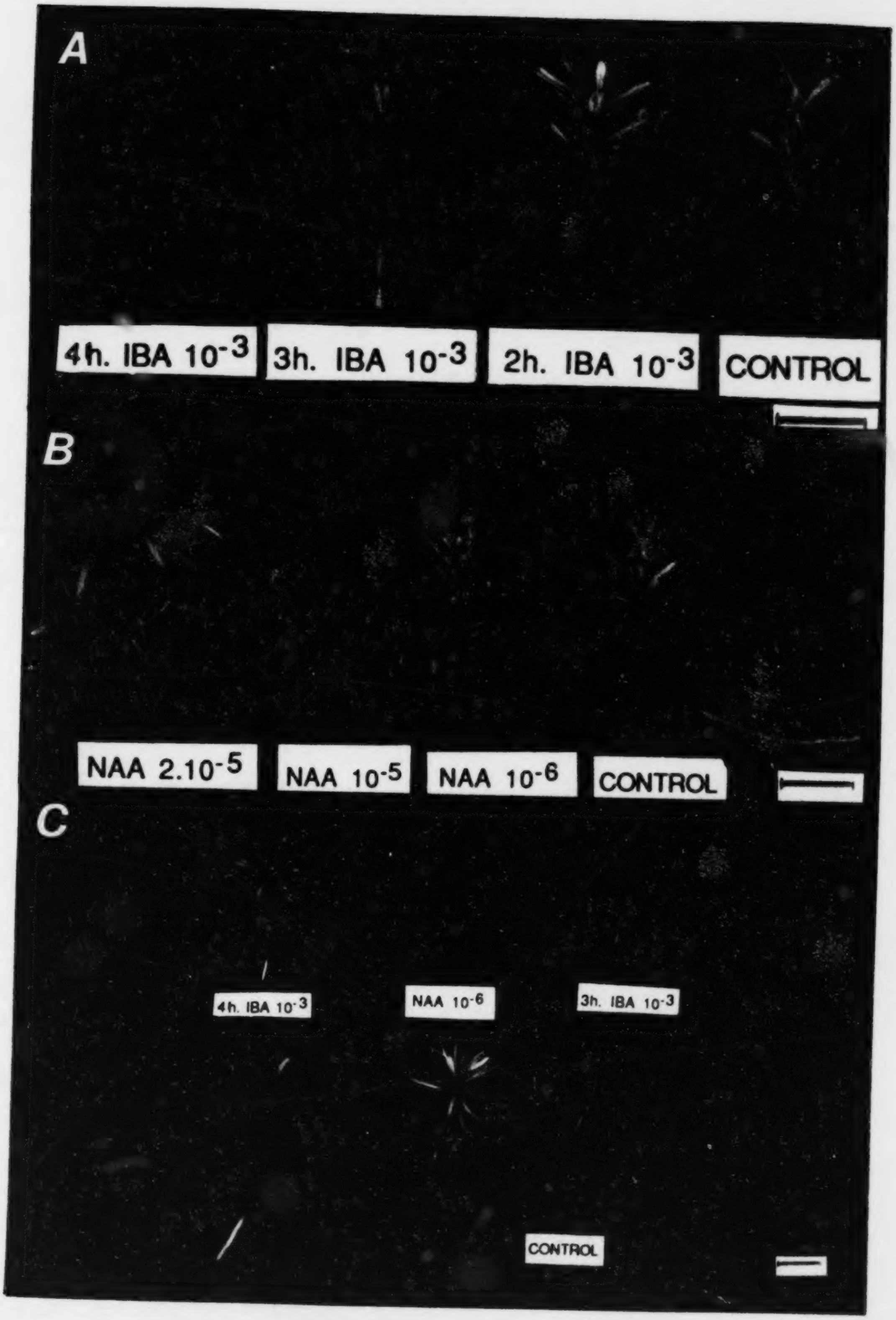
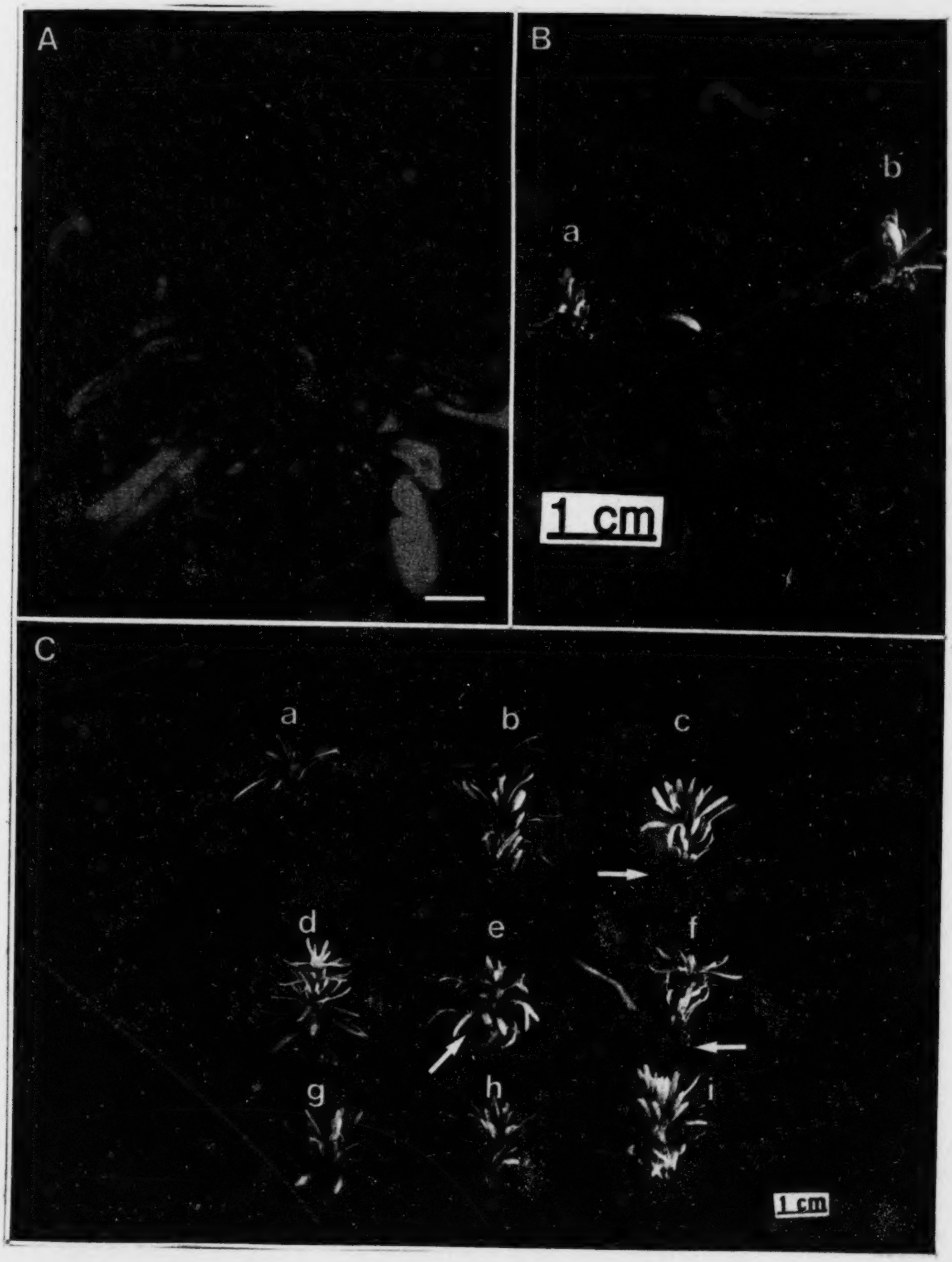

A
4h. IBA 10-3
3h. IBA 10-3
2h. IBA 10-3
CONTROL
B
NAA 2.10-5
NAA 10-5
NAA 10-6
CONTROL
C
4h. IBA 10-3
NAA 10-6
3h. IBA 10-3
CONTROL

Figure 12. Adventitious root formation on shoots of *A. amabilis* originated *in vitro.* After the initial treatment for root induction, the explants were cultivated *ex vitro* in a peat:perlite (1:1) substrate for a minimum of 4 months.

A. Adventitious shoot at a stage ready to be submitted to rooting treatment. Bar indicates 1 mm.

B. Spontaneous rooting in developing buds still attached to the original explant. Both figures were cultivated in 10μM BA during the first week and 0.1μM NAA or 2,4-D combined with 10μM BA during the second week. (a) adventitious root promoted by 0.1μM 2,4-D, and (b) adventitious root promoted by 0.1μM NAA for the second week in culture applied in identical conditions as (a).

C. Adventitious roots induced through different treatments and cultivated for 4 months under 1:1 peat:vermiculite, as follow: 9a) spontaneous rooting as described in A, after *ex vitro* treatment; (b) and (c) "pulsing" for 4 and 3 hours with 1mM BA and cultivated in the above soil substrate containing SH medium plus 30 mM sucrose; (d), (e) and (f) 20, 10 and 1μM NAA added to SH medium plus 2% sucrose in the sterilized soil substrate for 1 month, and then transferred to soil substrate just containing distilled water; (g) control cultivated with distilled water; (h) control cultivated with SH medium and 30mM sucrose; and (i) control cultivated with SH medium and 60mM sucrose.


A
B
a
b
1 cm
C
a
b
c
d
e
f
g
h
i
1 cm

allowed most of these explants to survive hardening for four months, while most of the adventitious shoots presenting roots failed to reach a hardening stage.

3. Embryogenesis

Somatic embryogenesis refers to a developmental process where cells form an organized bipolar structure displaying shoot and root poles connected by a functional vascular tissue. Embryogenesis can be either (i) direct, where somatic embryos originate directly from an explant without a callus phase or (ii) indirect, where somatic embryos originate after a proliferation of callus tissue (Thorpe, 1988).

Somatic embryogenesis proceeds, directly or indirectly, after the exposure of a potentially responsive explant to critical concentrations of exogeneously supplied hormones during the initial culture phase. Subsequent development of the immature somatic embryo into mature embryos comes after transfer to a secondary culture media, which may have a high osmotic potential or abscisic acid (Finklestein and Crouch, 1986; Hakman and von Arnold, 1985).

Attempts to repeat identical procedures to attain somatic embryos in *Picea glauca* and *P. mariana* (Lelu and Bornman, 1990) were also tried for *A. amabilis* (see p.42, item 3.1 and 3.2). Various kind of embryonic explants were employed in this approach, as demonstrated in section 3.1 of Materials and Methods (p. 42). Also variations of this method were performed using pulsing treatments with high concentrations of 2,4-D and BA or NAA and BA. Each auxin concentration was maintained at 0.1 mM and BA at 0.1 μM, as described in section 3.2 (p. 42).

Attempts to generate embryogenic tissue from small group of vegetative cells into somatic embryos have generated only nonembryogenic callus, and the best results are depicted in Figure 13.A and B. Initially, the 7-day old cotyledons, hypocotyls and epicotyl explants; when treated with 9.1μM 2,4-D and 4.5μM BA under continuos dark conditions, start to enlarge in both length and width, and callus initiation occurred within

3 to 4 weeks. Callus formation began on that part of the cotyledon in direct contact with the medium and eventually proceeded over the surface of the explants.

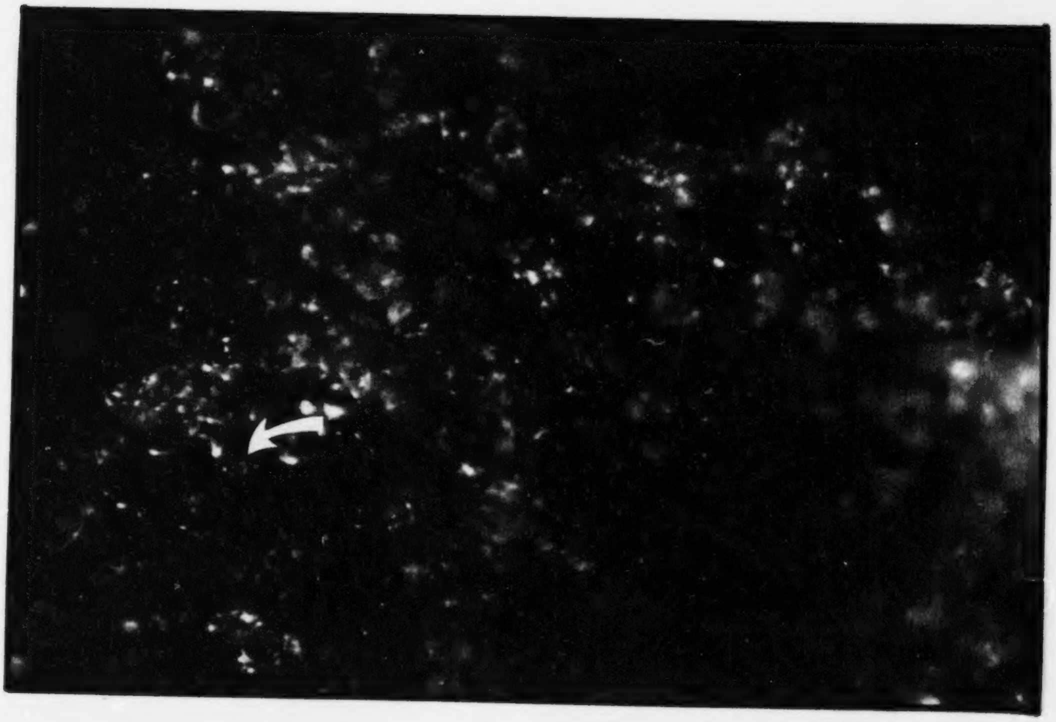
After continuos subculture on the same medium condition, proliferation of callus ranged in color from white to very light green and in texture from compact to friable. These cells appeared vitreous and high vacuolated when isolated individually (see fig. 13 at arrowhead). Also, after certain time in culture (4-6 weeks) these cells accumulated phenolic substances, started to turn brownish and became necrotic. Biweekly subculture on the same medium or a secondary culture media for embryo maturation did not prevent these cells from becoming necrotic.

Callus formation was more readily obtained in cotyledons and stayed longer than in other kind of explants. The cotyledon was the primary site of nonembryogenic callus induction with a frequency above 80%, while callus from hypocotyls and epicotyls reached lower percentages (±60%) and took longer to generate callus.

Also, pulsing epicotyl explants in filter-sterilized solution of equimolar concentrations of 0.1mM 2,4-D and BA for 24 hours and then transferring them to solid SH medium supplemented with 21.5μM NAA plus 0.45μM BA produced similar callus formation. After 5 to 6 weeks non-embryogenic callus could be distinguished coming out of the explant. All the callus formation were highly vacuolated. This callus eventually developed over the greater part of the explants' surface, and usually did not survive longer than 2 months in culture.

Finally, a third approach for generation of somatic embryos was also tried. In this case a range of auxins such as IAA, NAA, 2,4-D and IBA, with a range of cytokinins as BA, Z, K, and 2iP were experimented at concentrations of 1.0, 2.5, 5.0, 7.5 and 10μM. All possible combinations of one auxin and cytokinin were tried. Auxins were always maintained at the highest concentration. From these different combinations, NAA and 2,4-D when combined with BA (2.0, and 5.0μM) promoted more abundant callus. All callus obtained had similar characteristics with the nonembryogenic callus reported earlier.

Figure 13. Non-embryogenic callus formation coming froma treatment designed to induce somatic embryogenesis on 3-day-old epicotyls and cotyledons of *A. amabilis*. Arrows represents distinctive cells showing high degree of vacuolation coming from cotyledonary explants treated with 9.1μM 2,4-D and 4.5μM BA under continuos dark conditions. Subcultures were performed each month to identical culture medium. Similar formation appeared by pulsing epicotyl explants in filter-sterilized solution of equimolar concentrations of 0.1mM 2,4-D and BA for 24 hours and then transferred to solid SH medium supplemented with 21.5μM NAA plus 0.45μM BA. After pulsing treatment, all explants were maintained under dark conditions.



4. Histology, Histochemistry and Light Microscopy

Structural investigations have contributed significantly to plant tissue culture in areas such as organogenesis (Thorpe, 1980; Yeung *et al.*, 1981). Some of these advances are made possible through a better understanding of the stain action and new and improved microscopy methods. Successful application and interpretation of the staining provides better understanding of the plants' morphology, physiology and biochemistry (Jensen, 1962; Yeung, 1984). The main aims of these studies were to determine the target areas responding to application of phytohormones and to identify critical stages during the *de novo* organ formation in cotyledons of *A. amabilis.* These cotyledons were cultured in shoot-forming (SF) and non-shoot-forming (NSF) nutrient media from day 0 to day 24 and then fixed, embedded and sectioned as described in Material and Methods (p. 44-47). Also of importance was to put the sequential examination of the histological events in a temporal frame.

4.1. Histological Analysis of Adventitious Shoot Formation *in vitro*

Juvenile explants of pacific silver fir when cultured *in vitro* under aseptic conditions demonstrated a remarkable change in their pattern of development as a direct intervention of combinations of cytokinins integrated into a nutrient medium. In this case, a portion of the explant altered its pattern of development leading to *de novo* organ formation. A contrast to this pattern happens when the *in vitro* explants are cultivated continuously in a nutrient medium devoid of any phytohormones. In this case, the explants elongated or developed similarly to normal seedlings, and died following a certain degree of maturation or development. These features are referred to as shoot-forming (SF) and non-shoot-forming (NSF) depending on the presence or absence of cytokinins in the nutrient medium.

Cross sections of cotyledonary explants at the time of excision showed that cells located at or close to the epidermis facing the megagametophyte appeared morphologically different from those cells facing the cotyledons (fig. 14.B, C and D). These differences were mainly the presence or absence of resin canals and stomata. The differences in morphology did not preclude the *de novo* organ formation when these cells were put in contact with or under influence of combinations of cytokinins for certain period of time. At the time of the excision the cotyledonary explant measured ca. 3.5 to 4.5 mm and both epidermal and subepidermal cells were almost completely packed with food reserves (Fig. 14.A to D and 17.A).

When 3-day old cotyledonary explants were cultivated for a certain period of time in the presence of combination of phytohormones, the cells located primarily in the periphery of the inoculum in direct contact or adjacent to the nutritive medium became meristematic (see Fig. 15 A to F). The phytohormones responsible for this *de novo* process were constituted mainly by BA for the first week and combinations of cytokinins (Z+BA or K+BA) for the second week in culture. The newly dedifferentiated meristematic cells, characterized by distinct features, were capable of forming localized meristematic zones after 12 days in culture (fig. 15F). Later, after successive subcultures onto nutrient medium lacking phytohormones, these new meristematic zones turned into protruding domes (fig. 16 A and B). These meristematic zones were characterized by very active mitotic activity that eventually promoted the initiation and growth of the various organs that comprise a new plant. Finally, by the end of the organogenetic pathway, they achieved a stage similar to seedlings, presenting characteristic bipolar structures (apical shoots/apical roots), Figures 16C and 18C, respectively.

These patterns were considered the basic criteria to distinguish both systems (shoot-forming and non-shoot-forming systems) and to follow up the initial stages of the dedifferentiation process or the *de novo* organ formation in cotyledonary explants of *A. amabilis*. Mitotic activity in cotyledons cultured in the presence of combinations of cytokinins was not restricted to a specific region of the explant neither did it occur synchronously, however the general trend appeared as described above.

Figure 14. Light micrography of cross section of cotyledonary cells of *A. amabilis* at day of the excision (day #0).

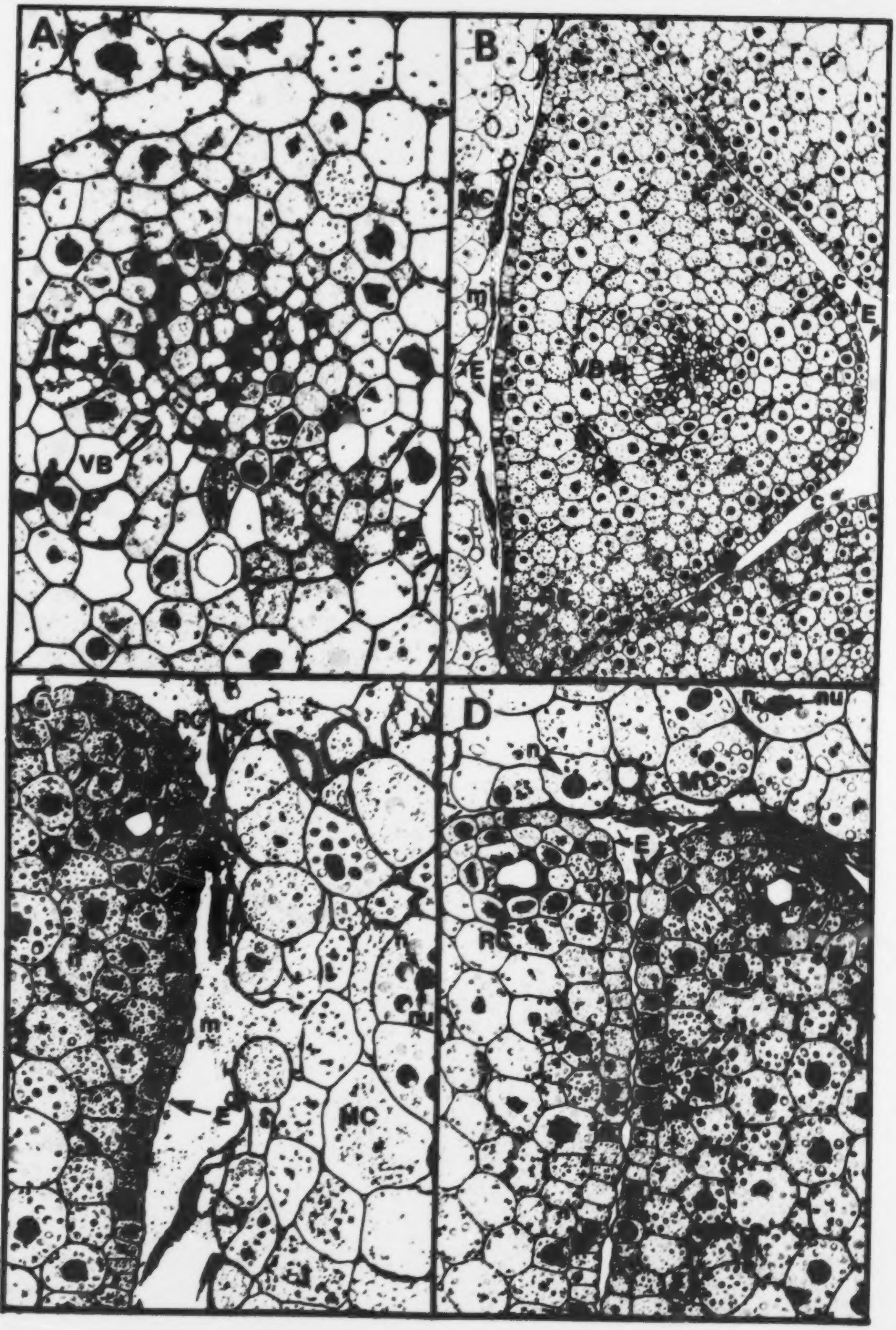
A. Light micrograph showing the vascular bundle at the center of the cotyledon surrounded by parenchymatous cells loaded with proteins, lipids and starch content. X 320.

B. Light micrograph showing the three different sides of a cotyledon at the time of excision. Surface m is in contact with the megagametophyte and is the region where stomata formation appears. Surfaces c are in contact with other cotyledons. X 125.

C. Light micrograph showing a more detailed view of side m, which is the one in close contact with the megagametophyte. X 320.

D. Light micrograph showing a more detailed view of side c. X 320.

Key: c, side of the cotyledon in contact with other cotyledon; E, epidermis; m, side of the cotyledon in contact with the megagametophyte; MC, megagametophytic cells; n, nucleus; nu, nucleolus; RC, resin canal; VB, vascular bundle.


A
VB
B
MC
m
E
VB
c
E
c
C
RC
m
E
MC
n
nu
D
n
nu
MC
E
RC
n

Asynchronous cell division was observed throughout the cotyledon explant. At first, for both systems, cell division occurred randomly following an initial anticlinal pattern, and this was more visible in the cells located at the periphery of the explant (see Fig. 15.A and Fig. 17.B). At days three and four in the presence of cytokinins, periclinal cell division was observed, also occurring in randomized form, but was found more concentrated in the epidermis and sub-epidermic region (parenchymatous cells) that were in close contact or adjacent to the nutrient media (Fig. 15.B). Within these cell layers, cell division was more pronounced, while the cells underneath gradually became vacuolated and non-meristematic.

Periclinal divisions also happened in regions that did not have a close contact with exogenous phytohormones, but these cases were less frequent. Also, close or near to the wounded base of the cotyledonary explant in contact with cytokinins was always characterized by a distinct and very active boundary of the meristematic zone and occasionally displayed an entire cluster of meristemoid cells, protruding out of the most external cell layer (Fig. 15.E). Up to day five and six, clusters of cells of periclinal and anticlinal origin appeared at preferential sites (Fig.15.C). These periclinally-dividing-cells possessed distinct features such as having an isodiametric shape and displaying a prominent and centrally located nucleus in most of which one, two or even more nucleoli could be seen (see arrows at Fig. 15C). A distinct boundary was also noticeable around these tightly packed cells. The nuclei of these cells also occupied a large portion of the cell's volume, and the vacuoles were basically small and distributed throughout the cytoplasm (Fig. 15.B at large arrows).

Differentiation of new organized structures start to appear mostly in the first and second layer of cells close to the epidermis. This formation was noticeable after five to six days in cytokinin-containing medium (Fig. 15.D). This pattern of cell formation termed *promeristemoid* (Villalobos, 1983; Villalobos *et al.*, 1985) has apparently originated

Figure 15. Light micrography of cotyledonary cells of *A. amabilis* at different stages of differentiation cultivated in combination of cytokinin-containing medium (BA+Z).

A. Cotyledonary cells at day #1. Note the highly stained cytoplasmic content, thinner cell wall and a centrally located prominent nucleus. X 320.

B. Cotyledonary cells at day #4, depicting the first periclinal division (large arrows). Note the intercellular spaces are small and stomata where chamber is absent. X 320.

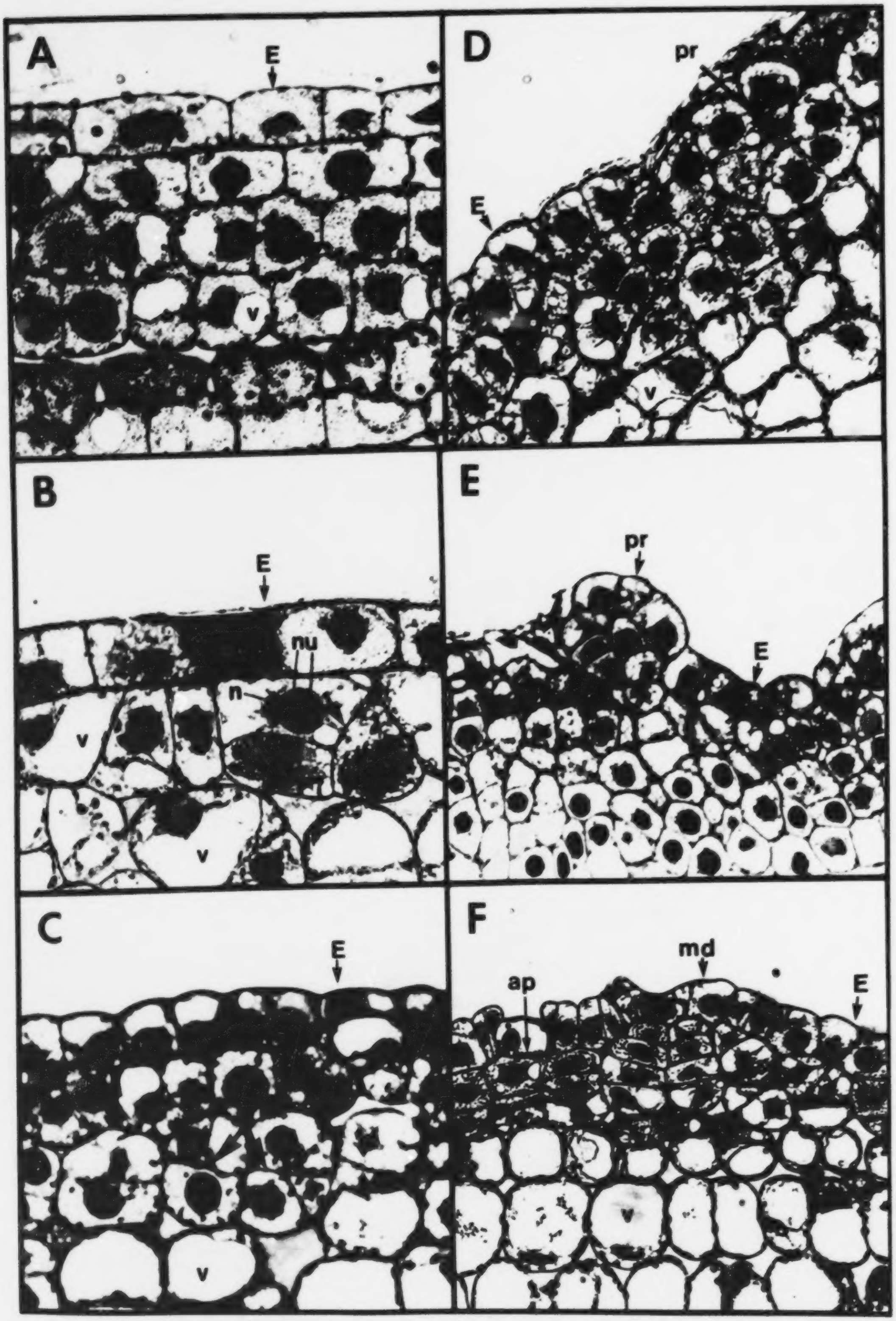
C. Cotyledonary cells at days #5, showing further divisions toward promeristemoid formation (see large arrows). X 320.

D. Promeristemoid differentiation in the first mesophyllic cell layer after 7 days in culture. Note the six-cells-formation in rounded shape, characteristic of the promeristemoid structure. X 320.

E. Promeristemoid formation at day #7 appearing at the base of the wounded cotyledonary cells. This event happens with less frequency. X 320.

F. Cells dividing to form the initial stages of meristematic zone formation at day #12 protruding from the epidermic and sub-epidermic cell layers. Note the small meristematic cells packed together and the suppression of stomatal structure containing starch grains. X 320.

Key: ap, amyloplasts; E, epidermis; md, meristematic dome; n, nucleus; nu, nucleolus; pr, promeristemoid; v, vacuole.


A
E
v
B
E
nu
n
v
v
C
E
v
D
pr
E
v
E
pr
E
F
md
ap
E
v

from a single small cell of rounded shape, whose genesis goes back to the first periclinal division at day three in culture (Fig. 15.B). Also, some time after the appearance of these new events (promeristemoids), suppression of stomatal development could be detected, as interpreted by the absence of stomatal chambers and by prominent starch grains inside the guard cells at day 12 (Fig. 15.F). These features remained in these conditions during the subsequent days in culture, and never were able to restore to their natural state as functioning stomata, even after transfering the explant to a medium devoid of cytokinins.

Another difference in shoot-forming cotyledonary cells in comparison to the control (NSF) was the persistence of a spherical or round-shaped nucleus located at or close to the central portion of the cytoplasm. These cells had a compact cytoplasmic content and thinner cell walls (Fig 15.C). The walls of these cells stained densely with PAS, indicative of increased deposition of cell wall polysaccharides. Also these cells showed intense staining of the cytoplasm and nuclei with Fuelgen and PAS counterstained with azure B indicative of high RNA and DNA concentrations (Fig. 15.B,C).

By the fourteenth to the eighteenth day in culture, periclinal divisions in the epidermal region, associated with a series of anticlinal and occasional periclinal mitotic partitions in the subepidermal region, provided the bulk of the cells for the developing primordia (Fig. 15.F). With subsequent development of subepidermal structures, elongation and addition of new cells in the epidermis had given the inoculum a nodulated shape (Fig. 16.A). As time elapsed these meristemoid clusters continued to divide and increase as protuding nodules. These organized structures proliferated extensively and gave rise to shoot primordia on the large hemispherical mass of meristematic tissue at the surface of the embryonic explant (Fig. 16.B). By day 24, small dome-formations containing needle primordia started protruding from the side of the cotyledon in contact with the medium. This new formation was visible with the naked eye. This dome-formation gave rise to the apical shoot with apical dome and needle primordia (Fig. 16.C).

On the other hand, cells cultivated in a cytokinin-free medium presented contrasting features (Fig. 17) with the previously described patterns (Fig. 15). The most remarkable and detectable anatomical differences were that in these cells the food reserves stored in the cytoplasm were rapidly depleted and they became highly vacuolated and displayed large intercellular spaces (Fig. 17 A to F). Also the cytoplasmic content of cell was confined to one side of the cell wall together with the nucleus. The plane of cell division was restricted only to anticlinal alignment, allowing the cotyledonary explant to enlarge in size mostly in one direction. Also these cells were bigger, showing with time an increase in the vacuole:cytoplasm ratio. The non-shoot-forming cotyledonary cells usually contained relatively small nuclei. Another notable feature was the presence of stomata distributed throughout the length of the cotyledonary explant. These stomata were visible in one side of the cotyledonary epidermal cells (Fig. 17.E,F).

After 4 weeks of culture of the explant in SF medium, distinct shoot buds with small needle primordia were evident. By this time each adventitious bud was developing a small vascular connection to the main vascular system of the explant. Once the vascular connection had been attained, the primary shoots developed rapidly with further growth and needle primordia formation. The control explants (cytokinin-free inoculum) on the other hand did not show any sign of *de novo* organ formation. The first sub-layer of cells from the epidermis which in contact with cytokinin had become meristematic and eventually lead to a completely new formation, in the non-shoot forming system have turned into a layer of palisade mesophyll followed by the spongy mesophyll cells which constituted previously the second sub-layer of cells. By this time they reached their maturation stage, with non-meristematic and highly vacuolated cells (Fig. 17F). Also, the formation of an epidermis, palisade mesophyll, spongy mesophyll, stele and resin canal was clearly visible with very distinct boundaries. Some time later these cells showed signs of necrosis.

Figure 16. Light micrography of longitudinal sections of cotyledonary cells of *A. amabilis* cultivated in presence of cytokinins-containing nutrient medium. The pictures are depicting regions of active mitotic activity from meristematic zone formation towards the final apical meristem structure achieved in elongating adventitious shoots.

A. light micrograph of the epidermal cells at day #14 showing the formation of a meristematic zone protruding from the epidermic and sub-epidermic cells. Note the suppression of stomata development. X125.

B. light micrograph of the meristematic dome formation at day #18. Note the suppression of the development of the resin canal. X 320.

C. light micrograph showing mid section of the apical meristem of an adventitious shoot at day # 49. X 320.

Key: am, apical meristem; md, meristematic dome; n, nucleus; nu, nucleolus; rc, resin canal; st, stomata cells; VS, vascular system.

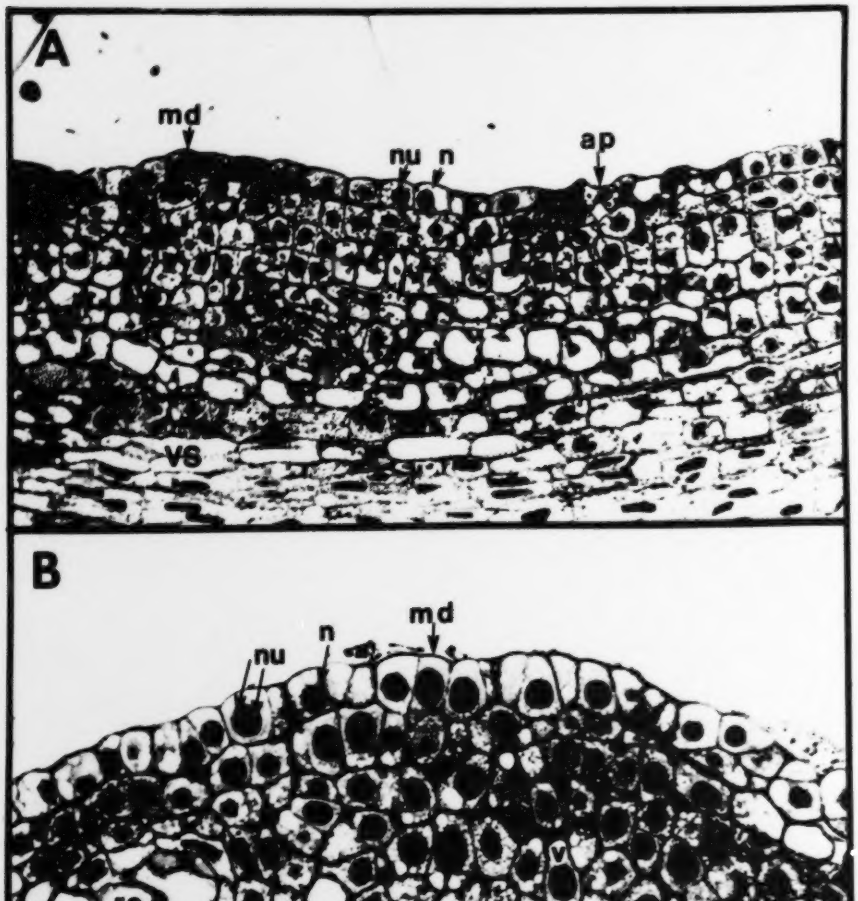

A
md
nu
n
ap
vs
B
nu
n
md
rc
v
C
am

Figure 17. Light micrography of cotyledonary cells of *A. amabilis* cultivated in the absence of phytohormones, showing distinct stages of differentiation.

A. cotyledonary cells at the time of excision (day #0). Note the intercellulary spaces at the second, third and fourth sub-epidermal cell layers. Also the same cells store a high content of reserve substances. X 320.

B. cotyledonary cells at day #1. Note the initial closure of intercellulary spaces with the start of cell division. The mitotic activity is performed only in anticlinal direction. X 320.

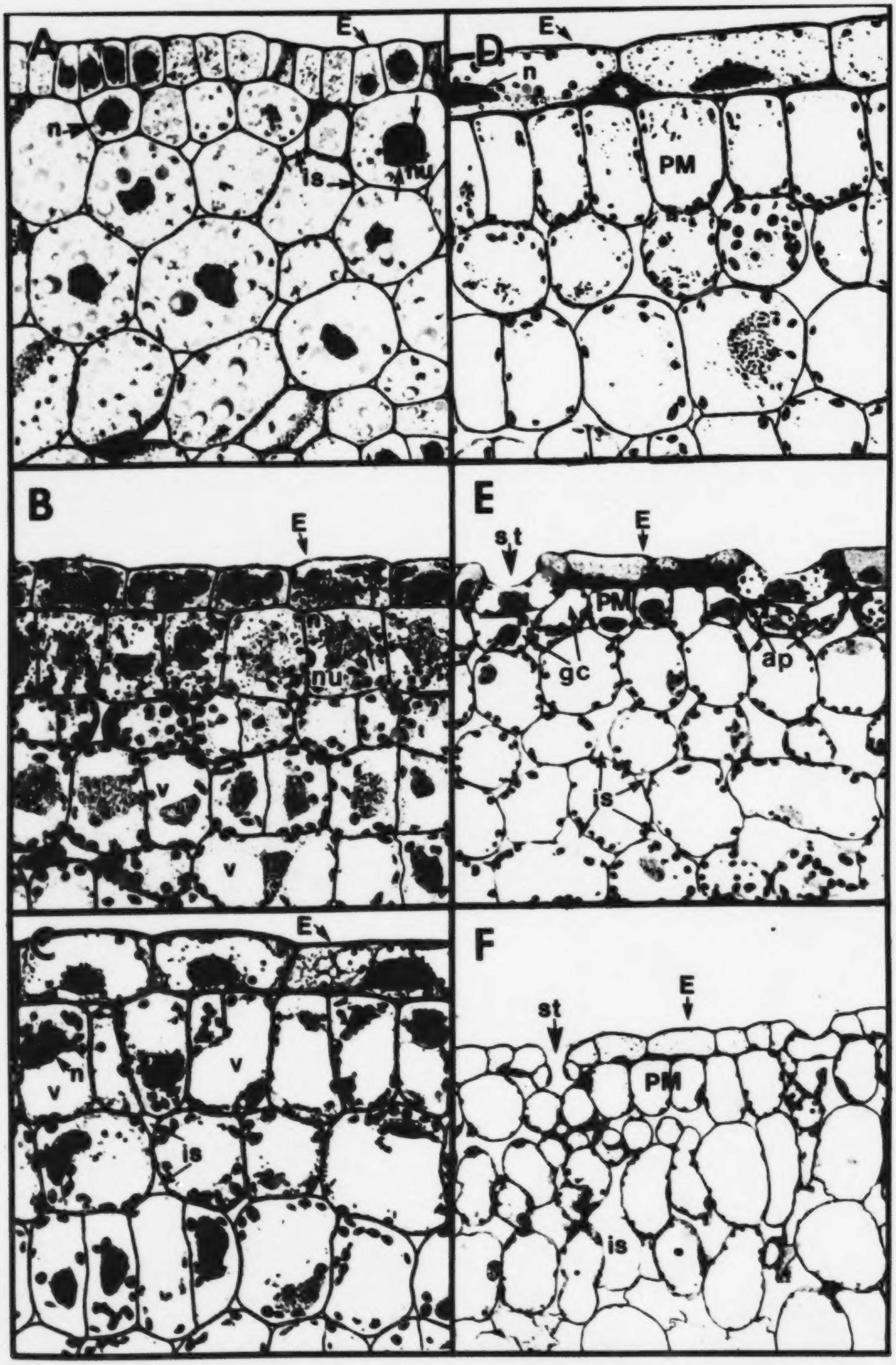
C. cotyledonary cells at day #4. Note the reappearance of intercellular spaces with the cessation of mitotic activity and emphasis on cell enlargement and depletion of reserve substances. Also the appearance of a large single vacuole and the concentration of the nucleus towards the cell membrane. X 320.

D. cotyledonary cells at day #7 showing further increment in intercellulary space and vacuolation. Note the differentiation cells of sub layers into palisade and spongy parenchyma. X 320.

E. cotyledonary cells at day #10 showing loss of cytoplasmic content. X 320.

F. cotyledonary cells at day #18. At this stage the intercellulary spaces are very well developed and sub-stomatal chamber is very prominent. X 320.

Key: ap, amyloplasts; E, epidermis; gc, guard cells; is, intercellulary spaces; n, nucleus; nu, nucleolus; PM, parenchyma mesophyllic; st, stomata; v, vacuole.


A
E
n
is
nu
B
E
n
nu
v
v
C
E
n
v
v
is
D
E
n
PM
E
st
E
PM
gc
ap
is
F
st
E
PM
is

In summary cotyledons destined to give rise to shoots showed certain distinct characteristics contrary to NSF cotyledons. Cells underwent dramatic changes, depending upon which conditions they were cultivated. For instance, those cotyledonary explants cultivated on shoot-forming medium usually showed four to five visible anatomical differences from those explants cultivated in a free-cytokinin medium. These distinctive characteristic could be summarized into the following observations:

1) Mitotic activity and plane of cell division;
2) suppression of stomatal development and resin canal;
3) restriction of intercellular space development;
4) promeristemoid formation.
5) cells' size and shape.

These differences are also summarized in Table 22.

4.2. Histological Analysis of *in vitro* and *in vivo* Adventitious Root Formation

Rooting *in vitro* and *ex-vitro* and hardening out the plantlets represent so far the two most challenging steps in the multistage organogenetic pathway of embryonic explants of *A. amabilis*. Nevertheless, as mentioned before, sometimes spontaneous rooting appeared while the plantlets were still attached to the original cotyledonary explant in the elongation phase (fig.12.B, p.115). This event occurs in about 6% of the adventitious shoots of specific treatments and probably reflected some sort of residual effect of auxin working in combination with cytokinins from the previous bud induction treatment. It was observed that those treatments that contained combinations of 2,4-D and BA, or NAA plus BA applied during bud induction phase showed these spontaneous rootings during the next bud development stage or still further during the elongation phase. This possible side effect of auxins alone or due to a synergistic effect of combination with cytokinin (BA) was still more visible before the adventitious shoots were submitted to a charcoal medium

Table 22. Features of epidermal and sub-epidermal cotyledonary cells of *A. amabilis* cultured in the presence or absence of cytokinins (BA + Z) during the first 12 days.

Cotyledon structure: at the time of excision (day #0) cotyledons measured ca. 3.5-4.5 mm, displayed compact nuclei (with visible nucleoli) and irregular outline and food reserves packed in the cytoplasm. The cytoplasm stained densely and reserve substances were abundant (starch, lipids and proteins). Cells in the epidermis were tightly pressed against one another and some were smaller than others. Very distinct difference between epidermis and subepidermic cells.

shoot-forming medium		non-shoot-forming medium
Centrally located nucleus with spherical shape occupying a large area of the cytoplasm. Food reserves abundantly distributed with extense reserve of protein and starch. Epidermis cells smaller than cells of the sub-layers (fig.17.A).	day #0	Identical features as mentioned in shoot-forming system.(Fig. 17.A)
Prominent spherical nuclei displayed in the center of the cell with small vacuoles. Cells densely packed together. Small intercellular spaces in the first sub-layers. Densely staining for starch and protein. (fig.15.A)	day #1	Numerous mitotic figures seen throughout the cotyledon. All cell divisions were anticlinal. Cells starting to enlarge. Small vacuoles aggregating (fig. 17.B).
First periclinal division seen. High mitotic activity. Small vacuoles. Nuclei centralized with one or two nucleoli. Densely staining for RNA and DNA. (fig.15.B)	day #3	Cells still dividing in lower degree. All divisions anticlinally. Vacuoles fusing and enlarging. Cell size increasing. Depletion of food reserves. (fig. 17.C).

(Cont'd).

Asynchronous cell division in anticlinal and periclinal directions. Cells meristematic, centrally located nuclei with a very rounded shape. Mitotic activity confined to epidermis and nearer layers. Meristemoids visible. (fig. 15.C,E)	day #5	Intercellular spaces more visible. Mitotic activity not detected. Cytoplasm confined to the cell periphery. Large vacuole and small nuclei near the cell wall. Mesophyll parenchyma differentiating. (fig. 17. D)
Non-meristematic cells highly vacuolated. Meristematic layer formed on the lower surface of the cotyledonary explant. Stomatal complex failed to differentiate. Meristemoid structure visible. (fig. 15.D)	day #7	Little noticeable changes from that reported earlier. Stomata complex completely differentiated. Epidermis palisade mesophyll, spongy mesophyll, stele and resin canal visible. (fig. 17.E)
Groups of prominent isodiametric cells formed. Periclinally and anticlinally cell division. Meristematic dome protruding. Stomata structures precluded. The lower cotyledonary layer became nodular. (fig. 15.F)	day #12	Little noticeable changes from that reported earlier. (fig. 17.F)

in the elongation stage. Charcoal is considered an important absorbent for traces of auxins or cytokinins still present in the explant. When these small plantlets showing spontaneous rooting were transferred to vermiculite:sphagnum (1:1) substrate, the root system normally continued to elongate (Fig. 12 C). Because the adventitious shoots have the capability to develop adventitious roots while still attached to original explant, this event presents potential in advancing our present understanding of rooting. This could be possible if we could identify which particular cells are the precursors of root primordia and what specific conditions are necessary to start this process. It could also be a valuable system for mass propagating *A. amabilis*, if understood, since a bipolar structure is formed almost concomitant to the bud induction process, therefore, it can minimize the painstaking and time-consuming steps of organogenesis.

Histological and histochemical studies conducted on adventitious shoots generating spontaneous roots, were designed to get insight into earlier events in cells undergoing *de novo* organogenesis. These early events were traced back to cells integrating with or surrounding the vascular cambium of the cotyledonary explant (Fig. 18.A,B,C). When specimen presenting spontaneous rooting were transversely sectioned in their bases, they showed a central pith core surrounded by vascular tissue and a cortex composed mainly of parenchyma cells loaded with starch (Fig. 18.A,B,C). These shoots undergoing spontaneous adventitious root formation showed the central cylinder with some kind of rearrangment in size, presumably due to an increase in cambium activity (Fig. 18.C,A). Figures 18.A and B probably represent a further stage of a process originated at figure 18.C. This feature contrasts with a non activated vascular cambium of the same specimen (Fig. 18.D at large arrowhead) and from roots of seedlings (Fig. 18.F at arrowhead). More advanced steps of the development of a root primordium could be traced to Figures 19.A, B and D. Also the parenchyma cells involving the vascular cylinder became loosely arranged in the cortical region and usually contained large amounts of starch (Fig. 19.A,B,D).

Light microscopy confirmed that in this process of new rearrangement and redirection of cells located in the swollen region, some of these cells accumulated phenolic substances (Fig. 19.B). It appears that some of the remaining cells that did not accumulate such substances were able to have an active role together with meristematic cells located at the vascular cambium that divided towards the epidermic cells. Since these cells appear to have originated from the vascular cambium of the cotyledonary explant (Fig. 18.C at large arrowhead) it is possible that in this specific process of the *de novo* root formation the meristemoid cells imitated with some variations the normal process of secondary rooting in normal plants (Fig. 18.E). It appears that a single cell or cluster of meristematic cells located at the cambium, after recognizing the differentiation signal started to divide periclinally, underwent a chain of reactions until giving rise to the root primordia at the epidermis layer (Fig. 18.C,A,B and 19.A,B,D). These meristematic cells were seen in different stages and probably represent some steps in root primordia formation (Fig. 18 and 19). Also tracheid nests were a constant feature close to or in connection with new adventitious roots and the central cylinder (Fig.19.C and D). Many of these tracheid nests had a vascular connection with the meristemoid cells or with the root primordia (Fig. 19.B). This spontaneous adventitious root formation had a smooth and distinct vascular connection between root and shoot (Fig. 20.B), and when transferred to *in vivo* conditions continue to develop as a normal plantlet, but some present symptoms of dormancy after few weeks into the new environment. Also spontaneus rooting was formed without the presence of too much callus formation, a feature very common in the process of adventitious root formation through the application of rooting agents.

In another study (figures not presented), using de-rooted seedlings that redeveloped new roots again as a control, an investigation was conducted to compare anatomical features between this control and adventitious roots in plantlets coming from cotyledonary explants. The de-rooted seedlings and adventitious shoots were apparently the same age and size and had identical rooting treatments. As expected, the newly rooted control had basically similar external features, such as callus formation at the base of the explant and produced fibrous roots at approximately the same frequency and time interval as adventitious shoots. The histological events did not show noticeable differences between systems, viz callus formation, large accumulation of ergastic substances, vascular cambium, xylem, phloem, and cambium-like cells. In addition to these tissues, the presence of tracheid nests were also observed and many of these nests developed very close to the vascular tissue or were even interconnected.

4.3. Histological Analysis of *in vitro* Root-Shoot Union

Histological studies of the vascular connection in the root-shoot junction of tissue culture-derived plantlets, de-rooted explants and seedlings of *A. amabilis* showed that in all plant types the xylem was well developed. The connection was smoother for the later than for the first two types. In all these plantlets, rooting that was initiated above the callus had direct vascular connections between shoot and root, but these were not as smooth and continuos as that normally found in seedlings. This difference did not preclude the normal growth of the plantlets until this stage. Figures 20.A, B and C show adventitious root connection with the vascular cylinder of the cotyledonary explant, and the meristematic portion of the adventitious root tip, respectively. All these pictures are related with spontaneous rooting formation.

Figure 18. Light micrography of transverse sections related to root formation in plantlets (A to D), and normal seedlings (E and F).

A. Light micrograph of a transverse section showing an agregate of meristematic cells containing densely staining cytoplasm and prominent nucleus. It is suggested that these meristematic cells could have an important role on further root primordia formation.

B. Magnified view of the meristematic cells shown in A. Note the constant presence of starch grains located mainly on the top and laterals of the meristematic cells. This cluster of active meristematic cells are located at the cortex tissue extending towards the epidermis. The origins of these cells is suggested to be at vascular cambium (see large arrow in the next picture). X 400.

C. Light micrograph showing a few concentrated meristematic cells emerging from the wounded cambium (large arrows). This indicated region could be implied to the starting point of root primordia formation X 125.

D. Light micrograph of a normal cambium cells in transversal section of expontaneous rooting in cotyledonary explants of *A. amabilis*. X 125.

E. Light micrograph showing the anatomy of a secondary root formation in seedlings. In this picture the vascular connection of the root is visible, smooth and regular. X 125.

F. Light micrograph of resting cambium cells of normal seedlings. X 125.

Key: CA, cambium cells; CC, cortical cells; MC, meristemoid cells; st, starch grains; XC, Xylem cells; WC, wounded cambium.

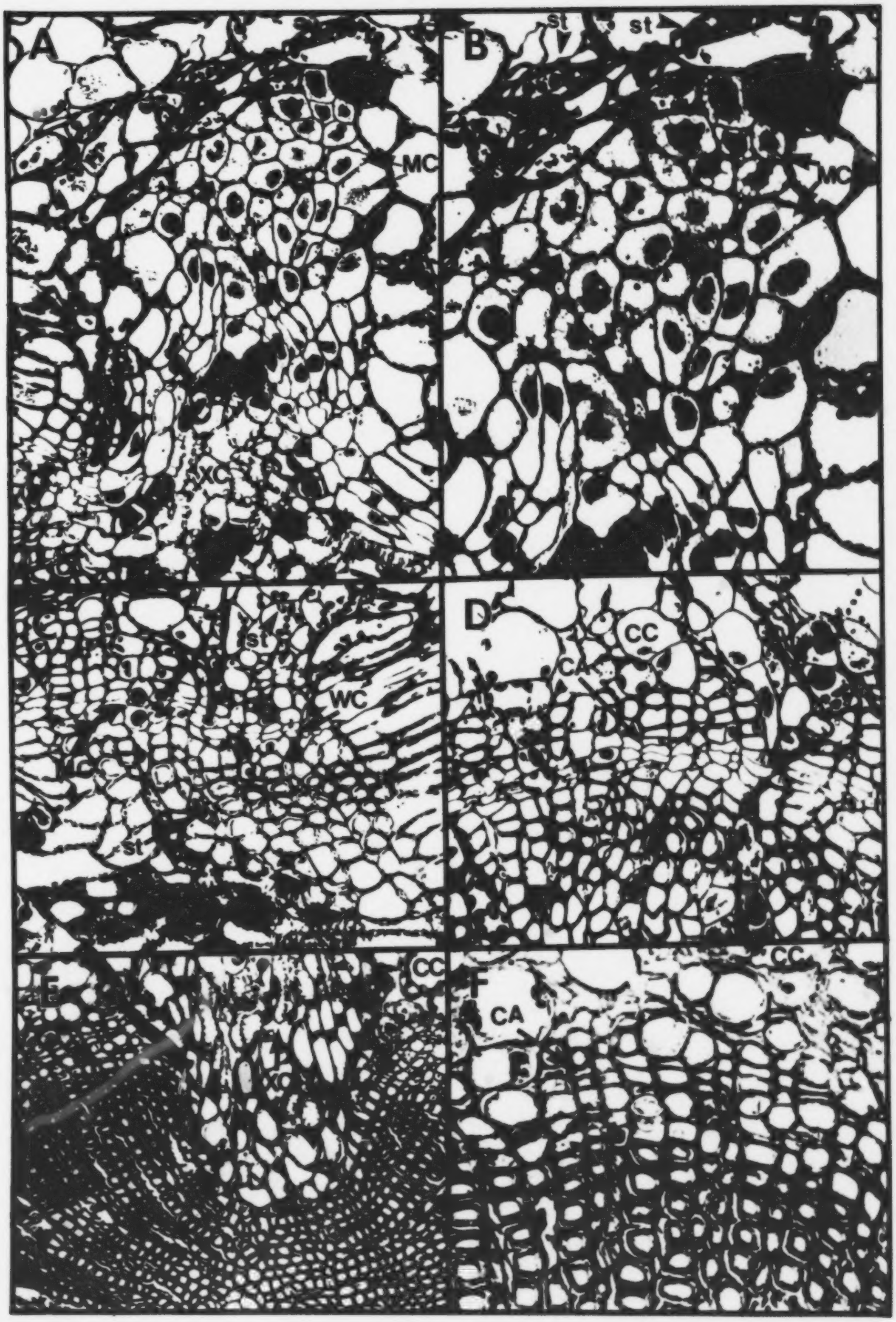

A
B
st
st
MC
MC
XC
D
st
WC
CC
CA
st
E
CC
F
CA
CC

Figure 19. Light micrography of transverse sections showing details of adventitious root formation in *in vitro* generated plantlets of *A. amabilis*. Accumulation of starch grains is a common feature in cortical cells.

A. Light micrograph of a group of meristematic cells in spherical shape protruding from the cambial region. These meristematic cells have high mitotic activity. Note the vascular connection between these meristematic cells and the vascular cylinder. X 125.

B. Light micrograph of an advanced stage of the root meristem going towards the epidermis and forming a visible meristematic dome. Note some xylematic tissue being developed between this meristematic dome and the tracheid nest located at the bottom of the picture. X 125.

C. Light micrograph displaying a magnified view of tracheid nests formation. Note the irregularly arranged tracheids being developed at the periphery of the vascular cylinder. A xylem arrangement is shown connecting the tracheid nests with the central cylinder. X 125.

D. Light micrograph presenting an adventitious root primordia formation. Note the constant presence of starch grains close to the actively dividing cells. Also see the high mitotic activity zone of cells at the root apical meristem and the tracheid nest in between the adventitious root primordia and the vascular cylinder. X 125.

Key: CA, cambial cells; CC, cortical cells; ES, ergastic substances; MC, meristemoid cells; RP, root primordia; RMC, root meristematic cells; st, starch grains; TN, tracheid nest; VC, vascular cylinder; XF, xylem formation; XY, xylem.

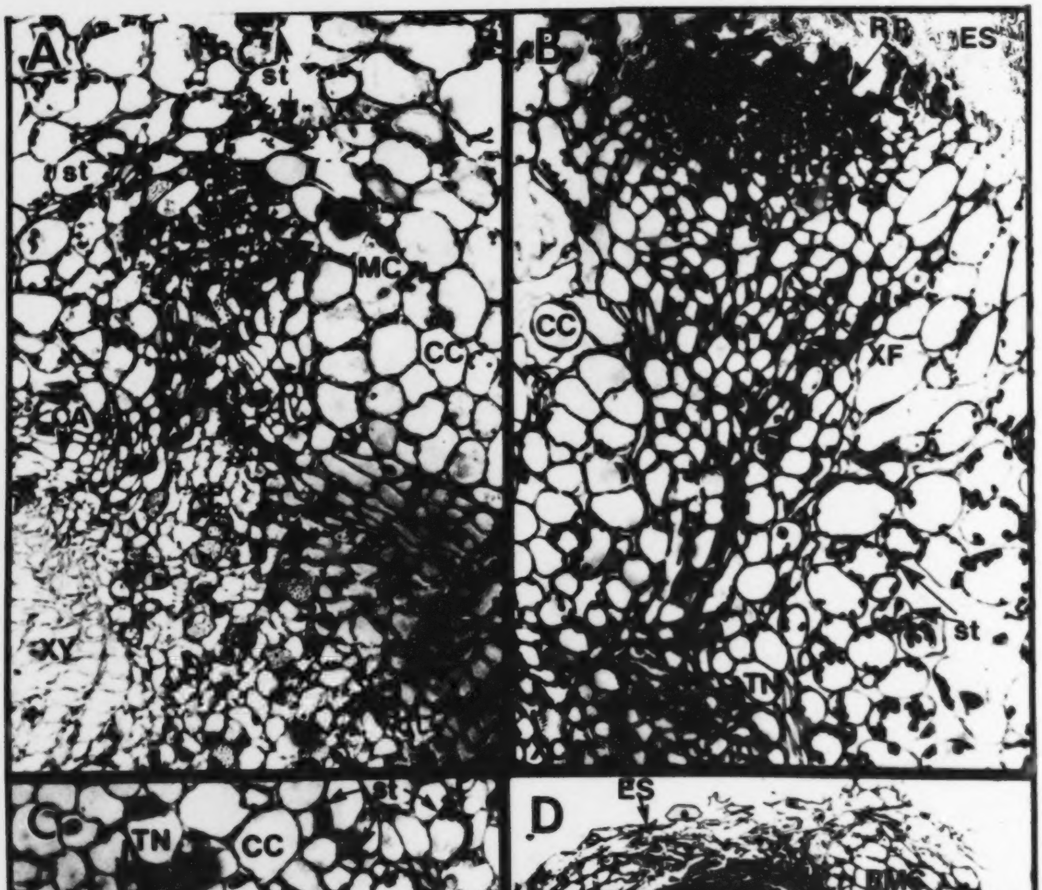

A
st
st
MC
CC
XY
B
RP
ES
CC
XF
st
TN
C
TN
CC
st
VC
D
ES
TN
CC
st
VC

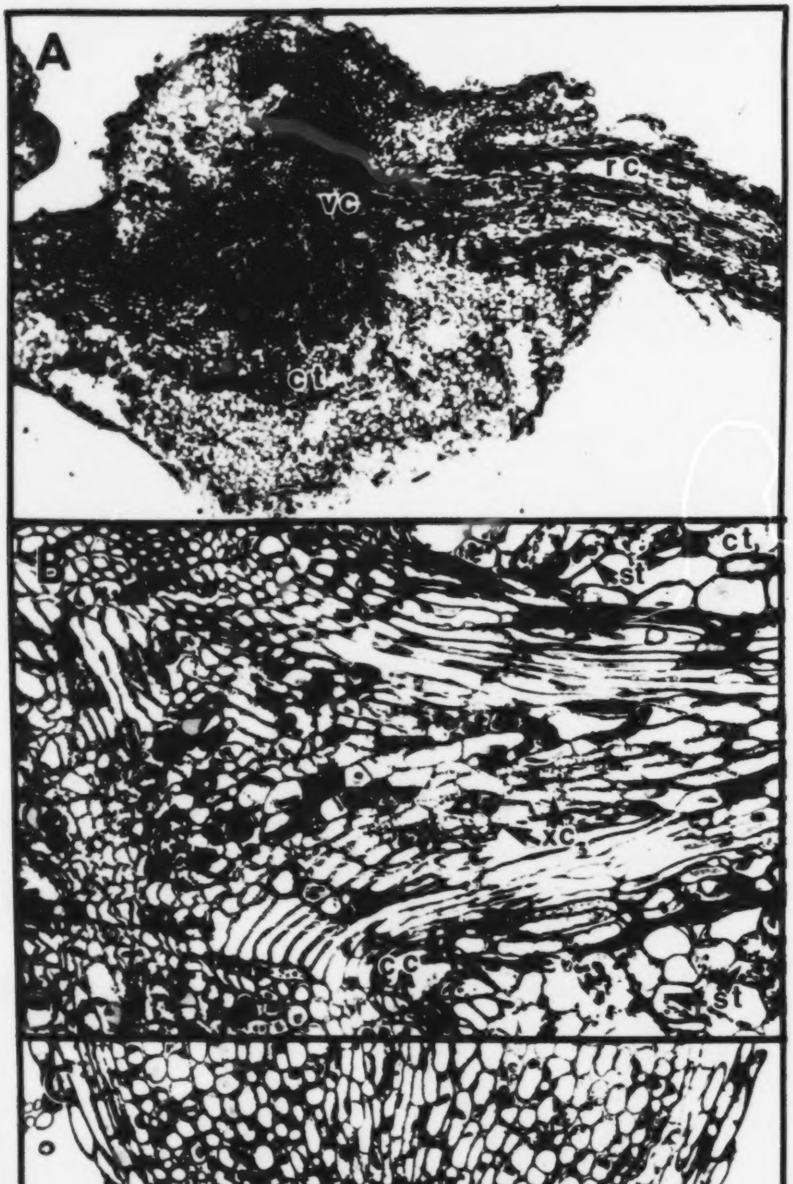
Figure 20. Light micrography of transverse sections showing the anatomy of the root-shoot connection of the tissue culture-derived plantlets of Pacific silver fir (*A. amabilis*).

A. Transverse section of the plantlet showing an overview of the root-shoot vascular connection. Note that the root emerges from the vascular cylinder and has a connection that goes from the vascular cylinder until the root tip. X 50.

B. Light micrograph showing a view of the root-shoot union. A magnified view of the root-shoot vascular connection is shown through a chain of xylem cells. X 125

C. Light micrograph of the apical root meristem. The root tip presents a well-organized structure and meristemetic cells showing mitotic activity. X 125.

Key: cc, cambial cells; ct, cortical tissue; rc, root cells; rmc, root meristematic cells; st, starch grains; vc, vascular cambium; xy, xylem cells.


A
rc
vc
ct
B
ct
st
xc
cc
st
C
rmc

V. GENERAL DISCUSSION

Tissue culture, as a biotechnological tool for micropropagation, is basically the reflection of two interpretative parameters: qualitative and quantitative. These parameters are affected by some degree of subjectivity, since they lack a distinct boundary and are difficult to be put into a mathematical format. Nevertheless they are a requirement for the completion of a new protocol, otherwise the interpretation would be incomplete. Thus both qualitative and quantitative measures were always taken.

1. Organogenesis

De novo organogenesis in plants has been accomplished in several species. This dissertation contributes with a new name to the short list of firs that are successfully micropropagated through an organogenetic pathway (see Table 1, Thorpe *et al.*, 1991). Although Pacific silver fir displayed many of the normal features of coniferous plants when micropropagated *in vitro*, it also presented some limitations for organogenesis not commonly found in its counterparts. For instance, while cotyledonary explants of many coniferous species respond to a wide spectrum of applications of cytokinins (Thorpe *et al.*, 1990), *A. amabilis* showed many restrictions to *in vitro* culture, as only a few juvenile cells responded and underwent the dedifferentiation process. This study shows that components of the culture medium and culture environment affected the morphogenic response of the explants cultivated *in vitro*, although the degree of response to the *de novo* organ formation varied between the different components. These factors when carefully optimized enhanced the number of competent cells responding to the dedifferentiation process. To get these competent cells of this recalcitrant fir to respond positively required an enormous amount of painstaking effort in labor and in time.

Some of the factors that allow for the dedifferentiation in cells of *A. amabilis* include age and size of the explant, type and concentration of gelling agent, sucrose, vitamins and salt formulation in the nutrient medium. Concentrations and time of exposure to cytokinins, as well as physiological treatments to speed up response and vigor of the explant were critical for the *de novo* organogenetic process. Although all of these factors appear to be common for other species, the main difference in *A. amabilis* is that it does not form adventitious buds unless a very specific combination of these factors is met. It appears that the requirements for the *de novo* organogenesis in this species are much more precise than for spruces and pines (T. Thorpe, personal communication), thus making the development of a micropropagation protocol very difficult.

1.1. Selection of the Explant

The selection of a suitable explant of *Abies* is the first priority for successful micropropagation. The development of an organogenetic protocol requires an inoculum that once selected must present the highest possible degree of homogeneity and be available for continuous experimentation. Using this approach, adventitious buds and shoots were obtained mainly from young cotyledonary explants and to a lesser extent from hypocotyl and epicotyl explants. However, this response is less pronounced than those reported for most conifers (Chandler and Thorpe, 1986; Harry *et al.*, 1987; Patel *et al.*, 1986; Pulido *et al.*, 1990). The developmental stages of these juvenile explants prior to culture greatly affected bud formation capacity (BFC) and shoot elongation capacity (SEC) indexes at single or at combinations of cytokinins. Cotyledons from 2-day old embryos germinated *in vitro* had not yet reached full bud-forming capacity potential, while 7-day old cotyledons had begun to lose regenerative capacity (Table 2, p.56 and fig. 1A, p.58). The best juvenile explant experimentally determined for bud induction was 3-day old cotyledons. A parallel 5x5 factorial experiment (Table 3, p.57) linking five salt formulations applied to different ages of cotyledonary explants indicated quite similar results. These results suggested a rapid decline in the morphogenetic capacity of the cotyledons related to their physiological age, and this is in agreement with Aitken-Christie *et al.*, 1985. An advantage of cotyledons over other embryonic explants is that they have a large surface area per volume. This factor provides that more cells of the explant can be in direct contact with the physical medium, allowing more competent cells to respond to the inducing factors.

Experience accumulated over the years has demonstrated that mass propagation of conifers, using tissue culture as a tool, has been overwhelmingly attained with juvenile explants (Thorpe, 1980; Thorpe and Biondi, 1981) since they present an unlimited theoretical potential of mass propagation rates (Thorpe *et al.*, 1990), and also because competence in totipotent cells decreases with maturity (Torrey, 1966; Murashige,1974; Brown and Thorpe, 1986).

1.2. Stratification

Submitting the sterilized seeds to 35 days of cold stratification seemed to improve significantly both quantitatively and qualitatively the number of adventitious shoots produced *in vitro* (Table 4, p. 61 and Fig. 1B, p. 58). In this regard, sulfuric acid played an important role in sterilization and scarification, and probably had an effect in promoting better hydration of the seeds, while they were under low temperature conditions. A parallel experiment conducted to correlate time of the cooling treatment and embryo vigor reinforced the above conclusion (Table 5, p.62 and Fig. 2, p.64), since the quantity of greener and vigorous embryos increased with time of stratification. Studies done on megagametophytes and embryos of lodgepole pine seed (Gifford et al., 1990) showed that during seed imbibition, the megagametophyte is involved in the translation of "stored" mRNA that could be involved in the limited hydrolysis of seed storage reserves. They mentioned the synthesis of three subsets of proteins from which one (possibly LEA) ceased translation prior to radicle emergence. Considering the few data available, it is possible that low temperature and moisture content enhanced the translation of hydrolytic enzymes in the megagametophyte and promoted invigoration of the embryos during the various times of stratification. However, this assumption can only be confirmed through specific study.

1.3. Nutrient Medium.

Nitrogen is the mineral element that plants require in the greatest amount since it is a structural component of proteins, nucleic acids, coenzymes, and numerous plant secondary products (Glass, 1988; Wolswinkel, 1988), therefore its absence is considered to be a major limitation to plant growth. The major differences between salt formulation employed in tissue culture lie in the amount and form of nitrogen plus the relative amount of some of the microelements (Gamborg *et al.* cited by Thorpe and Patel, 1984).

The response of cotyledonary explants of *A. amabilis* to bud induction and bud development was affected by the levels of macronutrients of different media (Table 6, p. 67). For all stages of the organogenetic pathway tested, Schenk and Hildebrandt's medium (1972) promoted the best average number of shoots per cotyledons. Further experimentation also showed that SH full-strength medium for the first two stages of bud induction followed by a SH half-strength salt concentration for bud development proved to be the best media for the first stage of *in vitro* micropropagation of *A. amabilis* embryonic explants. An examination of the major ions tested for tissue culture over the six different salt formulations tested (Table 1, p. 40) showed a substantial difference in the concentrations of nitrate, potassium, ammonium, and phosphate. A close scrutiny of the composition of these media used for the culture of plants reveals that they contain NO_3^- and NH_4^+, which are the sources of nitrogen for growth. Comparison between these six media influencing the *de novo* organ formation in this particular species of fir shows that there are quite large differences in the ability to promote bud induction and shoot elongation based on these different nutrient media and nitrogen sources (Table 6,p. 67).

One possible reason for superior bud induction and development on full- and half-strength salt concentration of SH medium might be the higher level of reduced nitrogen present in this medium compared to the other tested, except for MCM mineral salt formulation. However, results obtained with embryonic explants of *P. elliottii* suggested that *de novo* organ formation was limited by the presence of ammonium and high nitrogen concentrations (Pérez-Bermudez and Sommer, 1987), and others have also reported best results when ammonium was omitted or drastically reduced in the media (Sommer *et al.*, 1975; Konar and Singh,1980; Sommer and Caldas, 1981). These results could probably explain why MS and AE salt concentrations gave the lowest number of buds per cotyledons, as well as number of buds elongating and production of healthy shoots, although their average number of buds per cotyledon did not differ significantly from other salt concentrations. MS and AE nutrient medium have the highest concentrations of NO_3^- and NH_4^+. Perhaps *A. amabilis* is a very sensitive species that requires high concentrations of nitrate but low concentrations of ammonium. Also another factor that could contribute to enhance or restrain bud induction and development in this stage would possibly be changes in pH. This mainly happens in weakly buffered culture media, and when plants are grown on NO_3^- or NH_4^+ as a sole nitrogen source or even when this ratio is very unbalanced. During the growth of plant cells in media which are in general use, the pH of the medium changes; thus, it may drop as low as pH 4 and rise to pH 7. Such changes can be expected to have an impact on the metabolism of the cells (Dougall, 1972).

1.4. Concentrations of Cytokinins and Time of Exposure

Approximately 14 days in contact with combinations of two cytokinins seemed to be the optimal time for bud induction. Combinations of three cytokinins and extension of time of exposure in cytokinin above this optimum time did not improve bud induction in cotyledonary explants, instead they promoted callus formation. When 3-day old cotyledonary explants of *A. amabilis* were cultured in the absence of cytokinins, they continue to elongate and to develop towards their mature stage, but in the presence of combinations of BA plus zeatin or kinetin, the cotyledons formed numerous adventitious buds. Contrary to the general trend of one single cytokinin inducing *de novo* organ formation in most embryonic tissues of conifers (Reilly and Washer, 1977; Winton and

Verhagen, 1977; von Arnold and Eriksson, 1978; Rumary and Thorpe, 1984; Harry *et al.*, 1987; Pulido *et al.*, 1990), *A. amabilis* cotyledonary explants responded only to combinations of phytohormones and for a minimum period of 10 days in direct contact with these substances. Exceptions to this trend were also observed but they were very rare.

The assorted cytokinins and the different concentrations used were found to be the most critical component that induced adventitious bud formation. When a comparison was performed between the different cytokinins and auxins applied at the same time and in different levels, the cytokinin BA at 10μM showed superiority over the remaining phytohormones. This superiority was demonstrated by the fact that, with few exceptions, only BA was able to induce nodular tissue at the surface of the cotyledon, and few nodules turned into bud with needles. However, the great majority of these emerging buds became necrotic before turning into shoots, therefore this result indicated that some cells of the cotyledonary explant were competent to respond to BA, but somehow failed to develop further. After many subsequent experiments, it was realized that a combination of cytokinins was necessary for further development of the initial buds already formed. Combinations of two cytokinins showed better results than three cytokinins combined. The best combination of phytohormones to promote growth of the nodular formation was found to be BA with zeatin or kinetin at 10μM applied after the first week in the presence of BA alone. Combinations of BA with auxins for the second week in culture were extremely ineffective to promote elongation of buds.

It is important to note here that the establishment of *de novo* organ formation apparently had two distinct phases: first the permissive phytohormone combination was able to start the dedifferentiation process on target cells and the subsequently organogenetic tissue. The second stage was characterized as a capacity for continuous mitotic activity or lack of it on those newly formed meristematic tissue. It is obvious here that these combinations of BA and auxins were capable of promoting differentiation since they were able to induce a large number of adventitious buds for both treatments, but they somehow failed later in the subsequent stage of continous mitotic process. This evidence was corroborated also by histological and histochemical studies. Thorpe (1980) has reported that the endogenous auxin/cytokinin balance is a very important factor for the initiation of organogenesis, and also that the possibility of a certain interchangeability between auxins and cytokinins could be another factor, because cytokinins appear to have an effect on endogenous auxin levels. Work with *A. amabilis* has also confirmed the finding that in conifers, generally exogenous auxins is not required for organogenesis and is often counterproductive (Thorpe and Hasnain, 1988; Thorpe *et al.*, 1991).

Although the requirement for exogenous cytokinin has been well documented, few studies have been carried out on the metabolism and translocation of externally applied cytokinins. Also very little is known about the mechanisms of action of cytokinins and how they cause a response in plants, both at the cellular and the tissue levels, although cytokinins are known to be readily metabolized and interconverted in various forms. These various cytokinins compounds could be active in the cells through conjugation, hydrolysis, reduction or oxidation (Brock and Kaufman, 1991; McGaw, 1987).

1.5. Vitamins

Most plants are able to synthesize vitamins *in vitro*, and the question is raised frequently if additional vitamins should be added to the nutrient medium or whether vitamins are really necessary for plant tissue culture. Based on the results in this study it is clear that vitamins are important (Table 15, p. 84). Vitamins supplied at three times the standard concentration for the SH (Schenk and Hildebrandt, 1972) medium were beneficial for promoting the shoot elongation capacity (SEC) index in cotyledonary explants of *A. amabilis*. Although they were not critical for bud induction they appeared to be very important for bud elongation and enhanced the shoot vigor. These positive effects can be visualized in figure 4.A (p.85). SH vitamins are basically thiamine-HCl, nicotinic acid and pyrodoxine-HCl, which are the most common vitamins for different nutrient media. Vitamins have not been reported to be critical in tissue culture of woody species, probably because they have not been tested critically in most of the protocols. Based on this present study the question of vitamin requirements for *in vitro* culture in conifers, and perhaps other woody species, should be thoroughly examined.

1.6. Gelling Agents

Vitrification or hyperhydricity of the explants is a physiological condition leading to dark-green succulent abnormal tissue, resulting from reduced lignification and cuticle formation (Thorpe et al., 1991). It is especially common if the plant has too much water available, this being usually the case in liquid media, or if the medium has a low agar concentration (Debergh *et al.*, 1981; Aitken *et al.*, 1981). Vitrification of buds and shoots frequently occurs during *in vitro* culture, and there seems to be a multiplicity of factors involved in this phenomenon (Kevers *et al.*, 1984), such as high cytokinins levels, low irradiance and high temperatures, too intensive sterilization, softness of the explant and quality of gelling agents (Pierik, 1987). Furthermore, it has been reported that high cytokinin levels in combination with low agar concentration increase vitrification (Debergh *et al.*, 1981). This is a major problem since vitreous shoots do not survive transfer to *ex vitro* conditions (von Arnold and Eriksson, 1984), nor undergo elongation after bud induction treatment, as was the case of *A. amabilis*.

Agar is a seaweed derivative, obtained in pellet form, which can be used as a gelling agent in most nutrient media. This polysaccharide is easily the most expensive component of solid nutrient media, and when solubilized forms a gel that can bind water (the higher the agar concentration, the stronger the water is bound), and adsorbs compounds. During the development of a protocol for this species, several gelling agents were tried, namely Gelrite (also a polisaccharide), Difco-Bacto agar and Noble agar at various levels. Gelrite at low concentrations (0.2 and 0.25%) turned the explant vitreous and in increased levels the explant failed to respond to the *in vitro* conditions. Lower concentrations of a Difco-agar (0.6 and 0.7%) frequently made the nutrient medium appear sloppy, and so, bud induction was achieved infrequently. If a high concentration was chosen, then the nutrient medium became very solid, making inoculation difficult, and consequently, the inoculum also failed to respond. The optimum condition of solid medium was only achieved when Noble agar was added to the nutrient medium at 0.7%, since in this level the explant was able to respond and generate *de novo* shoot formation. Indeed, finding this level was a major breakthrough since until that point the nodular tissues protuding from the explant had generally failed to elongate.

Finding a suitable gelling agent together with the optimum combination of cytokinins referred to before, were considered the major factors that allowed the competent cells to undergo organized mitotic divisions continuously. Noble agar is a highly purified agar (most commercial agars contain organic and inorganic contaminants, as well as other impurities depending on their methods of manufacture). Probably embryonic explants of *A. amabilis* were especially sensitive to some of these contaminants. Agar contributes to the matrix potential, the humidity, and affects the availability of water and dissolved substances in the sealed container. Also concentration of agar or other gelling agents affects the availability of various medium components, in particular cytokinins (Debergh, 1983). The use of Noble agar for micropropagation in this species makes an expensive process even more expensive. It is clear that less expensive substitutes must be found if large scale micropropagation is to be feasible with *A. amabilis*.

1.7. Sucrose

The response of cotyledons to bud induction and bud elongation was dependent on the concentration of sucrose present in the medium. The average number of adventitious buds was maximum at 120 mM (4%) sucrose in the presence of cytokinins. However, when the numbers of shoots that effectively elongated above 5mm is included in this evaluation, 60 mM sucrose was better (Table 16, p.88). This same level was considered the optimum for bud development, after transfering the explants to a medium devoided of cytokinins. This interpretation is better demonstrated by comparing the quality of shoots produced against the different levels of sucrose, as depicted at figure 4B (p.85).

Concentration of 120 mM sucrose caused poor development of adventitious shoots, even if the optimum cytokinin concentration and sequence were maintained. Probably the osmotic stress due to the higher concentration of sucrose affected the ability to undergo the dedifferentiation process. Also, the elongation of the buds produced was reduced, since a large number of them were still nodular formation or small buds at the end of the experimental period. For most of the species micropropagated the energy requirement can be met in the range of 2-4%, although this can sometimes be replaced by glucose (Thorpe and Patel, 1984). For bud induction and elongation of western larch (Harry *et al.*, 1991) and black and white spruce (Rumary and Thorpe, 1984) sucrose concentration was found optimum at 2%, while for *in vitro* propagation of *Thuja occidentalis* (Harry *et al.*, 1987) and red spruce (Lu *et al.*, 1991) 3% was considered the best.

Organogenesis is a high energy-requiring process (Thorpe, 1990). This idea is supported by the relatively high accumulation of starch in regions of meristemoid cells, and its disappearance during subsequent phases of organogenesis. Histological studies of adventitious shoot and root formation in *A. amabilis* show large accumulation of starch grains in the cells behind those committed to the *de novo* process (Fig. 18, p.139 and Fig. 19, p.142). These reserves of carbohydrates are there probably due to the high demand of energy. Therefore, while they are being consumed, an additional supply of carbohydrate must be continously supplied, especially during the heterotrophic phase of plantlet formation. Carbohydrate has also been shown to act osmotically during organogenesis (Thorpe, 1980; 1983). The relative importance of exogenous carbohydrate for these two roles in *A. amabilis* was not investigated.

Recent review has shown the mechanisms of transformation or breakdown of monomeric saccharides or polysaccharides in aqueous solutions at high temperature (Schenk *et al.*, 1991). One of the main breakdown products of monomeric saccharides, especially hexose, is 5-(hydroxymethyl)-2-furaldahyde (HMF) which is known to be biologically toxic (Moye, 1964; Weatherhead *et al.*, 1978 - cited by Schenk *et al.*, 1991), and at elevated temperature *circa* of 15 to 25% of the sucrose may be hydrolyzed to glucose and fructose, components which can be further synthesized to HMF or phenolics. It is expected also that this hydrolysis of a dissacharide into two monosaccharides will promote some alterations on the osmotic potential of the nutrient medium. This information indicates that beside the fact that sucrose acts in a dual role as osmoticum or energy requirement in tissue culture, it could also be a source of toxic products to the explant depending on autoclaving procedure, although this aspect was not investigated in this dissertation.

1.8. Liquid Pulsing

Administration of BA as a liquid pulse for short periods of time was found to be more efficient in promoting *de novo* organogenesis in epicotyl and hypocotyledonary explants than the conventional method of incorporation of BA into the solid medium. Pulsing 21-day old epicotyls and 7-day old hypocotyls with 1mM BA was very effective for induction of adventitious bud and shoot development, although the number of adventitious shoots produced were less when compared with cotyledon cultivated in solid medium (compare Tables 18, p.94 and 19, p.95 with Table 17, p.90). Although the number of adventitious buds per epicotyl or hypocotyl is significantly higher than those obtained for cotyledons cultivated in solid medium, each embryo produced an average of 4-5 cotyledons which makes them more atractive as an inoculum for mass propagation.

A period of 3 hours for 21-days old epicotyls and 2 hours for 7-days old hypocotyls with continuos agitation was sufficient for maximum response in these explants. Longer exposure periods showed no increase in the frequency or quality of bud induction. Cotyledons of different age when exposed in pulsing treatment, even for short time, formed a mass of nodular tissue that aggregated latter and grow into a mass of callus (Fig. 6, p.96 and Fig. 7, p.98). Probably one reason for this is that cotyledons are small explants (± 4.0 mm) and so most of their cells will became saturated immediately and show sensitivity to high levels of cytokinin. Perhaps a large range of pulsing time and concentrations of BA could provide an alternative protocol for cotyledonary explants of *A. amabilis* and reduce the long time required to generate elongated shoots. This in turn would reduce the number of steps and thus the cost of the micropropagation process.

1.9. Shoot Elongation

When shoots were approximately 5 mm long they were excised from the original explant. This generally required 5 to 8 subcultures. Bud elongation was best achieved in the absence of phytohormones, while maintaining the previous levels of the remaining ingredients, with the exception of SH salts formulation which were cut by half. After 8 weeks in culture, separating the shoots 5 mm high from the mother explant and transferring the elongating buds into a half-strength DCR major salts formulation containing 0.05% activated charcoal and the 60 mM sucrose resulted in the optimum *in vitro* conditions for elongation of buds into shoots (Table 20, p.102). The mode of action of activated charcoal is not completely understood, but it acts as an important filter and is added to the medium with the main purpose of absorbing phenolic substances, as well as any trace of phytohormones (Friborg *et al.*, 1978). In this treatment, the average 5 mm buds more than doubled their sizes within 2 months in culture. Subculturing was performed bi-weekly. Similar results were obtained for a quarter-strength SH medium (Table 21, p. 103), and the qualitative factor of the shoots can be visualized in Figure 8 (p.104). These results agree with most protocols for woody species where a reduction of major salts are a requirement for stem elongation (Thorpe and Patel, 1984). Also reduction of the concentration of sucrose for the elongation stage is a common feature in many conifer protocols. In *Pinus radiata* (Aitken-Christie and Thorpe, 1984), *P. contorta* (Patel and Thorpe, 1984), *Picea glauca* and *P. mariana* (Rumary and Thorpe, 1984), *P. rigida* (Patel *at al.*, 1986), *Pinus canariensis* (Pulido *et al.*, 1990) a sucrose concentration of 2% was optimal, but for *Thuja occidentalis* (Harry *et al.*, 1987) 3% was the optimum. In *A. amabilis* 2% sucrose was considered the best concentration for shoot elongation.

1.10. Shoot Multiplication

Unlike results on shoot multiplication of many conifers, adventitious shoots of *A. amabilis* failed to generate a second wave of adventitious buds coming from elongated shoots. However, the potential number of axillary buds generated naturally in each shoot 3 cm high could triple the original number of shoots (Fig. 10, p. 107). If these preformed axillary buds are allowed to develop and form shoots, the number of true-to-type plants could be tripled during this stage. This lack of effectiveness could result from the size of shoots employed (Indra Harry personal communication). Shoots bigger than 4 or 5 cm should reverse this trend, but plagiotropic growth could still be a problem since results obtained from axillary buds generated spontaneously in elongating shoots failed to grow orthotropically. The inability to achieve shoot multiplication in *A. amabilis* significantly reduces the chances of developing a large-scale protocol for clonal propagation of this species. Further studies on this problem are needed.

1.11. Adventitious Root Formation

Promotion of adventitious root on shoots taller than 1.0 cm originating from cotyledonary explants was attained in a very low percentage. Also the process itself is very slow and takes a long time to be accomplished (usually 5-6 months). This limited the number of experiments that could be carried out in a reasonable period of time.

Furthermore this length of time is at least double that needed for rooting for other temperate conifers, e.g, *Picea glauca* and *P. mariana* (Rumary and Thorpe, 1984) and *Larix occidentalis* (Harry et al., 1991). So far the best results appeared when the auxin IBA, was included to the rooting treatment. Pulsing in sterilized solution of 1mM IBA for 3 and 4 hours and plating the shoots on a SH medium supplemented with 30mM sucrose gave the best results for rooting. In these cases, respectively 14 and 17% of the adventitious shoots rooted, but the quality of rooting differed in both treatments (Fig. 12C, p. 114). Also, treating the explants with NAA at 10 and 1μM in SH nutrient medium with 60mM sucrose promoted root initials, but the quality of the roots was poor when compared with pulsing in IBA, and the percentage of response for these treatments did not reach 10%. All other treatments applied without nutrient medium and sucrose failed to produce adventitous roots, and the shoots turned yellowish after a certain period of time in the soil substrate. The soil substrate was composed of perlite:vermiculite (1:1) and was sterilized by autoclaving prior to use. In contrast, spontaneous rooting at a frequency of about 6% occurred on some shoots, while they were still attached to the cotyledonary explant. This appeared to be a consequence of previous treatment, as it only occurred when auxin was used with cytokinin in the bud induction stage.

Although a maximum of only 17% of the shoots formed roots, this compares favourably with previous findings with Fraser fir, in which 18% rooting was achieved (Saravitz *et al.*, 1991). In Fraser fir all plantlets subsequently died. With *A. amabilis*, the spontaneously produced plantlets also died some 2-3 months following separation. The induced-rooted plantlets survived up to 9-10 months, and appeared healthy, but were lost along other rooted material due to contamination. Whether these plantlets were viable or not, therefore, could not be determined, as no hardening studies could be carried out. Histological studies did not indicate any structural problems of the roots or their connection to the shoots (see later). Rooting, and to a lesser extent hardening, remain the most significant problems in the development of a micropropagation protocol in conifers (Mohammed and Vidaver, 1988). As seen here much more work is needed on this topic.

Much physiological and biochemical research has been aimed at gaining insight regarding post-translational processes of rooting including the influences of plant growth regulators on metabolite levels. These studies were focused on those factors that may foster or curb required processes for rooting. But the the fundamental biology of this important developmental phenomenon is still obscure (Haissig *et al.*, 1992). It is expected that progress in understanding the control of rooting will be improved through the availability of new technology for studying plant developmental phenomena, especially those that go closer to the gene level.

2. Embryogenesis

Haberlandt in 1902 postulated that cells from any plant, given the appropriate stimuli and proper conditions, could be induced to regenerate plants (cited by Thorpe and Brown, 1986), and since the development of somatic embryos in carrot cells, numerous species have been regenerated *in vitro* by this method (Ammirato, 1983). The large majority of plants regenerated through parasexual methods belong to the angiosperms. Very few species of gymnosperms have successfully achieved a protocol for mass propagation through somatic embryogenesis. This is due to the fact that at the present time our understanding of the stimuli and conditions necessary for induction and control of the somatic process is minimal. A key factor in the successes achieved so far has been the choice of explant (Lu and Thorpe, 1987; Dunstan, 1988; Thorpe, 1988). In one of the latest lists of somatic embryos induced in conifers shows that most of the explants came from immature embryos (Tautorus *et al.*, 1991). Lu and Thorpe (1987) presented data on the potential for somatic embryogenesis relative to the stage of development of immature and mature embryos of *Picea glauca*. The potential for the induction of somatic embryos from mature seeds was shown to vary with the geographical provenance, the number of years that seeds had been stored, and the imbibition time of seeds (Tremblay, 1990).

Mature seeds of *Abies amabilis* were found to be recalcitrant for *in vitro* techniques aimed at induction of somatic embryos. Only non-embryogenic callus was obtained. Besides the fact that mature seeds were the source of inoculum, it is difficult to know if this recalcitrance is partially due to *Abies* species being more primitive in terms of evolution when compared with *Picea* species, in which somatic embryos from different sources of explants has been obtained. *Abies alba* and *A. nordmanniana* were reported generating embryonic tissue in vitro (mainly from megagametophyte and immature embryos), but failed to undergo subsequent plantlet production (Table 1. Tautorus *et al.*, 1991). The fact that more research has focused on *Picea* species, since *Picea abies* was the first species in which somatic embryos was successfully reported, could account for some of these differences.

Somatic embryos occur in a repressed state of development while on their induction media, and usually have required passage onto a differentiation medium to obtain further maturation. This has, in some cases, involved the removal of phytohormones from the induction medium, while in others ABA has been used (Dunstan, 1988). Also, the recovery of plantlets from somatic embryos is presently very poor and remains a limitation to the implementation of this technology (Dunstan, 1988).

3. Histology, Histochemistry and Light Microscopy

Histological and histochemical studies were carried out with cotyledonary explants to gain a better understanding of the early events leading to the *de novo* organ formation in *A. amabilis,* and to arrange all information into a chronological sequence to provide additional insights for this recalcitrant species. From the vast array of literature, it is well established that exogenous cytokinins are a pre-requisite to assist totipotent cells to dedifferentiate. However, the regulation and timing of the early events during the *de novo* bud formation are not well known. The main reasons for this are that i) only a few cells are involved in bud initiation, ii) the initial cells cannot be easily identified, although the dedifferentiation process occurs mainly at the epidermal and subepidermal layers and, iii) the bud differentiation process is usually asynchronous. This asynchrony also affects bud induction and development, as well as shoot elongation.

In this dissertation it has been shown that combinations of cytokinins were an absolute requirement for inducing totipotent parenchymatic cells to divide and form active meristematic tissue that eventually lead to shoot primordia formation. The whole series of events leading to these changes from normal cells committed to be tissues like epidermis, mesophyll parenchyma, resin canals or stomata into a totally different new function, takes place in a very restricted region. So, a natural question would be: where is this restricted area located? The obvious answer will point to the region where would-be normal parenchymatic cells turn into meristematic cells. Histochemical examination revealed that these cells showed intense staining of the cytoplasm and nuclei with Feulgen and PAS counterstained with azure B, indicative of high RNA and DNA concentrations (Fig. 15.B,C). Also PAS reacted with the contents of the cell wall suggesting increased deposition of polyssacharides. This active region of cells are located mainly in the epidermis and the two subsequent rows of mesophyll parenchyma cells, that were in close contact with cytokinin-supplemented media. Also the cells located towards the basipetal region of the explant were more responsive than the ones located at the acropetal portion. The possible explanation could be that in the acropetal region, the cells are relatively older or more mature when compared to the cells located at the base of the cotyledonary explant (Dale, 1992). This would create a gradient of physiological age and so the response to the cytokinins would be expected to be lesser due to the uneven state of differentiation. When the explant was plated horizontally on the surface of the shoot forming medium, the aforementioned rows of cells became meristematic and showed intense mitotic activity. This very active region was more visible at day 3 in culture. The places where these events occur in *A. amabilis* are in agreement with previous work done with *P. radiata* (Yeung *et al.*, 1981; Villalobos *et al.*, 1985).

Although in most of the coniferous species a single cytokinin has been demonstrated to be sufficient to send signal(s) redirecting the target cells to become meristematic, in *A. amabilis* this was rarely the case. Apparently, the message addressed by a single cytokinin onto cotyledonary cells did not send strong enough signals to commit totipotent cells to form adventitious shoots. Instead, some target cells apparently received and interpreted the signal, and started to divide following a new pattern, but stopped just after few mitotic divisions. Sometimes these divisions led to the formation of small "bumps" in the surface of the cotyledons, but seldom reached more advanced stages.

Dedifferentiation involves a series of events, starting from induction and leading finally into a complete change in the fate or destiny of a cell or group of cells (Christianson and Warnick, 1983). Considering the whole process of *de novo* organ formation in *A. amabilis*, it appears that there are initially two different developmental phases that if successfully attained will eventually lead to more advanced stages of shoot formation. The first phase is mainly related to processes of induction and dedifferentiation, starting with the day of excision and appearing to close with the formation of an organized cluster of at least six cells after 7-8 days in culture. This cluster of organized meristematic cells in rounded shape and presenting a distinct boundary was termed promeristemoid (Villalobos *et al.*, 1985) and represents the most advanced stage of differentiation during the initial phase (see Fig. 15.D, p.125). Although this description of dedifferentiating cells was made following observed sequential stages, it does not reflect complete chronological synchrony in the explant since different stages were seen in the same cotyledon and in cotyledons of different ages than those mentioned for the sequential steps. Although these results are in accordance with previous work done in radiata pine by Yeung *et al.*, (1981) and Villalobos *et al.*, (1985), the second stage has shown remarkable differences. This phase started approximately at the end of promeristemoid formation, and required a combination of high concentrations of cytokinins (equimolar concentration of BA and kinetin or BA and zeatin) otherwise the process of organ formation would not undergo further development. This phase initiated at day 8 in culture when the explants were subcultured onto a combination of cytokinin-containing nutrient medium, and was maintained for several days after the transfer of the inoculum to a shoot elongation medium. It is important to emphasize here again that without the addition of the aforementioned combinations of cytokinins (BA + Z or BA + K) the number of adventitious shoots formed were quite low, and the few that elongated struggled to survive, showing in most cases abnormal growth or dormancy. Cotyledonary explants cultivated just in a nutrient medium devoid of phytohomones never led to *de novo* organ formation. Since cytokinins are an absolute requirement for the *de novo* morphogenic process, it seems that some of their function could be the prevention of cotyledonary cells from elongation, and induction of adventitious shoots through the establishment of meristemoids (Villalobos *et al.*, 1985).

Observations at the time of excision also revealed that the three sides of the cotyledonary explants possessed a few anatomical differences. These differences were more visible at the epidermal cells, where resin canal and potential stomatal cells were located on the side in close contact with the megagametophyte (Fig. 14, p. 122). So this raises the question as to whether the nearby environment could be one of the possible explanations for these differences. In contrast to the other two sides of the cotyledon in close contact with each other, the side facing the megagametophyte has the potential to have food reserves more directly available, or be exposed to other factors (Fig.14.B). Depending on the specificity of medium conditions, in which the inoculum was plated e.g., non-shoot forming and shoot-forming medium, those specific sides of the cotyledonary explant presented contrasting anatomical features, such as presence or absence of stomatal structures, palisade mesophyll, spongy mesophyll and resin ducts physiologically functioning or not.

At the time of culture, the cotyledons were plated arbitrarily and on those sides that were cultured in direct contact with the cytokinin-containing medium by coincidence, all the normal anatomical features resembling stomata and resin canals became inhibited or suppressed by day 5 in culture. Also, the cotyledonary surface proximal to the megagametophyte had not developed any stomata at the time of excision (Fig. 14.C,D, p.122), and it appears that when this side coincided to be in direct contact with cytokinin-containing medium, the potential stomatal structures did not differentiate further, since it was observed that some shoot-forming cotyledonary faces showed a few inhibited and sunken stomatal structures (Fig.14.F, p.122), while in others stomatal structures were not detected.

In shoot-forming systems of cultivated Pacific silver fir cotyledonary explants, organized differentiation generally occured in the first rows of cells in direct contact with the medium, which could be due to a possible physiological and biochemical gradient of nutrient substances and phytohormones coming from the medium into the cells. The suggestion that such gradient could determine the position of *loci* at which organized structures would rise (Ross and Thorpe, 1973) could possibly help to explain the process of dedifferentiation *in vitro*. The initiation of these organized structures is not synchronous for every explant, but usually it starts at day 3 or 4 in culture with some cells showing mitotic division in a periclinal direction. Subsequent anticlinal divisions occurr in the daughter cells proximal to the epidermis, forming a 4- to 5-celled structure and appearing mostly at days 5-6. At this stage, the entire epidermal and mesophyll layers of cells became meristematic and committed to continuous division. By days 7-8, the first meristemoids were completely formed. These resulted from cell division in a radial direction resulting in its typical spherical shape and thus ending the first stage of this *de novo* process for this system. In all of these histological studies so far done for this dissertation, it shows a strong affinity with the idea that for *de novo* organ formation, probably a single cell starts to divide periclinally fostering organized development of a polar structure (Thorpe, 1980).

Histological and histochemical studies were also accomplished to assess the quality of root-shoot connection, type of vascular tissue involved, smoothness of the connection and anatomical features of the adventitious roots developed spontaneous or after induction. Also a very careful and time consuming study was undertaken to get a better insight about time and tissues involved in the early events of adventitious root primordia formation, and if possible to ascertain the very cells committed to this *de novo* process.

Adventitious root formation in cotyledonary explants of *A. amabilis* has been observed emerging in adventitious shoots following two patterns: i) in small shoots (≤ 5mm) while they were still attached to the cotyledonary explant; and ii) as a result of pulse treatment in high concentrations of IBA (1mM) for 3 and 4 hours, or low concentrations of NAA. In this case, IBA induced at first a swollen region at the base of the shoots, from which a small amount of callus was formed later on the process of rooting.

Slide preparations for the latter specimens demonstrated that basically all the adventitious roots were initiated above the callus formed, and therefore there was good vascular connection between the root and shoot. In the former case those adventitious roots formed spontaneously showed good connection and were capable of initial continuous development, when the plantlet was transferred to *in vivo* conditions. These spontaneously formed roots were the object of detailed histological and histochemical studies.

The interpretation of many mounted slides brought some additional insights into the dedifferentiation process of spontaneous root formation for this species. Adventitious root initiation in cotyledonary explants appears to proceed from observable changes coming apparently from a single cell or perhaps a very limited number of cells which is/are conducive to meristemoid cell formation subsequently (Fig.18, p.139 and 19, p.142). Although sectioned tissues represent discrete stages in an organogenetic process which is continuous, there are dangers in concluding that an event observed at some point in time precedes or succedes a second event observed at later or earlier time at the same tissue location in a different specimen. In my understanding this problem will appear constantly in this kind of work. However, if the events placed earlier in the chronology of adventitious root formation have been observed with a relative degree of frequency and if they appear specifically in those specimens committed to *de novo* organ formation there must be at least some degree of certainty in describing the events in a sequence. Secondly, this degree of certainty could be increased if in the same specimen many *de novo* organ are being formed asynchronously in different parts of the inoculum; thus allowing one to compare possible sequential events. Thirdly, when for the same organ or tissue a standard pattern is compared showing different formation(s) there must be at least some degree of accuracy in saying that the rare feature has something to do with the dedifferentiation process under investigation. Although this is not conclusive proof, it cannot also be disproved categorically. Finally, considering all these points I feel comfortable in interpreting what I have observed, although more in-depth studies would be necessary to characterize the distinct stages for this *de novo* organ formation. Biological material rarely develops exactly and synchronously as human beings would like to have them, and sometimes this can be taken as a positive factor in analyzing the object under investigation, since sometimes it provides different stages of organ formation within the same explant. Considering the literature available, with some exceptions (Smith and Thorpe, 1975), it appears that the detection of a single cell responsible for meristemoid development is not reported; although Torrey (1966) also suggested that typically a single cell in a mass of cells is somehow activated and undergoes a series of cell division culminating in the formation of meristemoids or meristematic tissues. However, he presented no evidence to support his view.

4. Data Interpretation

The basic assumptions for application of analysis of variance (ANOVA) are that (1) the observations should be independent, (2) the errors normally distributed, and (3) the error variances constant among groups. Besides these basic requirements, randomization and replication are implicit in this statistical analysis. Since these assumptions were met (Gisele Engels and Tak Funk-University of Calgary, personal communication) analysis of variance (ANOVA) was the statistical tool selected to calculate whether there were significant differences among treatments, and Duncan Multiple Range Test was employed *a priori* to make specific comparisons between the treatments. When unplanned comparisons or specific comparison were done *a posteriori*, Scheffé's test was the choice, since in this case the probability of erroneously rejecting the null hypothesis at a significant level of 0.05 is greater than 5%. Also, these approaches turned out to be more robust since all the treatments were based on equal sample sizes.

The interpretation of the raw data for the majority of the factors affecting organogenensis shows a trend, rather than definite or conclusive results. This trend is visualized when significant differences appear between the various treatments, although in many cases the factors studied did not show a gradual effect, as measured by the numbers of buds produced or by the number of shoots elongating. The physiological state of the cells represented by their different ages in Table 2 (p. 56) is a very important factor in determining the competency of totipotent cells to undergo dedifferentiation. Age also reflects the capacity of the explant to respond to the application of different salt formulations supplemented with phytohormones. This response is visible in the form of induction and elongation of adventitious buds as a component of BFC index, as demonstrated in Table 3 (p. 57) and Table 5, p. 62). Besides the aging effect, composition and molarity of salt components of most nutrient medium studied also had an impact on organogenesis, and this is shown in Table 3 (p. 57) and specifically at Table 6 (p. 67). Time of stratification or cooling treatment also had an impact in increasing the number of competent cells responding to the effect of phytohormones, as depicted at Table 4 (p. 61) and Table 5 (p. 62). Significant differences were also found in the kind, concentration and mode of application of single or combinations of auxins and cytokinins as displayed at Table 8 (p. 71) and Table 11 (p. 75) Also time of application, concentration and period of exposure in cytokinins is critical for induction and development of adventitious shoots in embryonic explants (see Table 7, P. 69).

The application of SH vitamins other than the standard concentration appears to have an effect in reducing the average number of adventitious buds per cotyledon, although it was found only few differences between the different levels of vitamin dosage. A significant result appeared when the treatments were compared for BFC, showing a continous trend in decreasing the capacity for bud formation as the dose of vitamins increase. Paradoxically, SEC showed a different result, where three times the normal dose of SH vitamins was considered the best treatment for achieving elongated shoots. This is a tipical situation for *in vitro* micropropagation of Pacific Silver fir where the best results for average number of shoots per explant and BFC index, do not represent the optimum conditon for SEC index. Also, the ultimate product (elongated shoots ready for rooting) sometimes does not correlate to the best induction treatment, since the buds generated do not elongate in a synchronous fashion.

Sucrose level was critical in affecting the average number of buds per cotyledon and it also affected BFC index (Table 16, p. 88). This same factor had a strong impact on shoot elongation, while salt formulations had a minor contribution (Table 20, p. 102, and Table 21, p. 103). Periods of "pulsing" treatments in high concentrations of filter-sterilized solutions of 1mM BA were found to be highly significant and can be an effective factor for promoting bud induction in embryonic explants of *A. amabilis*.

Finally, although an overview of the statistical analysis is important in a way that it shows a mosaic of effects due to different levels of critical factors and explains some of the variability found between the various components responsible for the *de novo* organ formation process, the final decision must be based upon the balance of the qualitative and quantitative parameters for mass propagation *in vitro*. Thus the development of a micropropagation protocol for a particular conifer is likely to remain an empirical one, in which the effects of the various factors must be determined systematically. Furthermore, the effects of any treatment on the final product must also be kept in mind. The interplay of subjective (qualitative) and objective (quantitative and statistical) parameters in the development of a micropropagation protocol has been reinforced by this study with *A. amabilis*.

5. Summary

Using the protocol described in this dissertation, 59% cotyledonary response was obtained when 3-day-old cotyledons were cultured for 7 days on 10 μM BA and another 7 days on equimolar concentration of 10 μM BA and zeatin or BA and kinetin. The cotyledons were subcultured on 1/2 SH after the first two weeks in cytokinins for a period of 10 to 12 weeks. Subcultures were done bi-weekly. Excised shoots were elongated on 1/4 SH (or 1/2 DCR) for an additional period of 12 weeks. In these conditions, 94% of the buds succeeded in elongating and turning into shoots 10 to 15 mm high. Seventeen percent rooting was obtained by culturing 24-week-old shoots by pulsing in a filter-sterilized solution of 1mM IBA for 4 hours and plating the shoots in a perlite:vermiculite (1:1) substrate supplemented with SH medium plus 2% sucrose. The rooting process took 5-6 months.

From each seed, an average of 5 cotyledons were obtained. Of these, 3.5 formed about three to ten shoots each when all optimum conditions were used. After 19 weeks in culture, approximately 16 shoots were greater than 10 mm in height. After three additional subcultures these shoots rooted yielding about 2.7 plantlets per seed. The major problem encountered was that of rooting the *in vitro* generated shoots. Adventitious shoots 15 mm high failed to remultiply at that size, but they have the potential to yield a threefold increase due to the presence of axillary buds at that stage. Thus this protocol is of limited value at present.

The anatomical events culminating in shoot formation included high mitotic activity, suppression of stomatal development and resin canal formation, restriction of intercellular space development and differentiation of organized structures. These organized structures included anticlinal and periclinal mitotic figures, meristemoid formation, meristematic domes and juvenile leaf primordia. Shoot primordia and root primordia arose from single cell(s) in the subepidermal layer and vascular cambium respectively. The plantlets had solid root-shoot junctions, and mid-sections of root and shoots portrayed normal features.

The laboratory-scale protocol described needs to be improved before scale-up can be attempted for possible large-scale clonal propagation. Nevertheless it presents potential for intermediate purposes, such as multiplication of seeds from disease-free trees and for seed orchard development. Despite the limited number of plantlets produced per seed in this present study, it represents a more successful protocol than previously worked for any member of the *Abies* species. The work carried out in this dissertation thus reinforces the view that *Abies* species is the most difficult to work with under *in vitro* conditions.

VI. CONCLUSIONS AND RECOMMENDATIONS

The results presented in this dissertation demonstrate a reproducible system for generating adventitious shoots *in vitro* from juvenile explants of Pacific silver fir. This organogenic approach involved the empirical multistep pathway employed to determine the best conditions required for each step, from the induction of competent cells to dedifferentiate until polar shoot formation, generated in the explants. A series of experiments were undertaken with the objectives, of developing a protocol for *in vitro* propagation, through organogenesis and embryogenesis, in *A. amabilis* (Dougl.) Forbes and thus building up the groundwork for research in operational vegetative propagation utilizing somatic tissues from trees in adult growth phase. Also a contribution was aimed at getting a better understanding of the possibilities and constraints of applying this protocol into commercial reforestation or seed orchard areas. Finally a better understanding of the *de novo* process of adventitious shoot and root formation, and the establishment of a reliable timetable of the histological events was also sought. The combination of different approaches allowed for the observation and assessment of those identified goals. Furthermore, the number of potential plantlets formed via the protocol developed here is greater than previous studies with *Abies* species. This study has led to the following conclusions:

I. In *A. amabilis* cotyledonary system, application of a single cytokinin (BA) during the first 7 days in culture and combination of equimolar concentrations of cytokinins (BA and K or BA and Z) for the subsequent week allowed the induction of the best average number of adventitious shoot production. In this system 3 to 7 potentially rootable shoots could be generated in 59% of the explants, which allows an estimate of 12 to 28 adventitious shoots per embryo.

II. The best embryonic explant was found to be 4.5 mm long cotyledons obtained from cultivated embryos plated in sucrose-agar medium for two days in darkness under cold temperature (2°C) and three days in light conditions at a temperature of 24 ±1°C.

III. Full and half-strength of SH salt formulation (Schenk and Hildebrandt, 1972) were optimum for bud induction and development of the adventitious buds generated. Both concentrations were supplemented with 60 mM sucrose, three-times the SH vitamins recommended, and 0.7% Noble agar®. The pH was adjusted to 5.7-5.8 before autoclaving.

IV. Shoot elongation was best achieved at half-strength DCR salt formulation (Gupta and Durzan, 1985) or a quarter-strength SH (Schenk and Hildebrandt, 1972) salt formulation supplemented with 0.05% activated charcoal and maintain all of the remaining ingredients.

V. Multiplication was not achieved, nevertheless the 3-4 potential axillary buds were formed in earlier stages.

VI. Rooting was achieved in very low percentage (17%) and took a long time approximately 5-6 months.

VII. Histological and histochemical studies showed similar patterns to previous work (Yeung *et al.*, 1981; Villalobos *et al.*, 1985). Promeristemoid formation, described by Villalobos *et al.*, (1985), is derived from a single or a group of subepidermal cells, and appears completely formed by day six to seven in culture in a medium supplemented by cytokinins as referred to in item I.

VIII. Cellular differences between shoot- and non-shoot-forming systems were observed after the first day in culture. The former presented: 1) large area of meristematic cells with actively dividing cells showing dense cytoplasmic content and smaller vacuoles, 2) suppression of stomatal development, 3) restriction of intercellular space development, and 4) differentiation of organized structures.

IX. Generation of adventitious roots directly in cotyledonary explants could be traced back to cells of the vascular cambium, which are activated by internal agents (residual effects of auxins or auxins combined with cytokinins). This activation generated meristematic cells that started to divide periclinally. These meristematic cells after sucessive mitotic activity find their way towards the epidermis. Tracheid nests, resin canal and parenchymatic cells loaded with reserve nutrients may play an active role in this process.

X. The organogenetic processes responsible for the *de novo* formation in adventitious roots and shoots could have some similarities and it appears that the genesis of both processes could be explained by a single or a small group of totipotent cell(s) that receive(s) a new signal and start(s) to divide periclinally. This has never been proved conclusively, neither disproved categorically.

These findings, taken together, indicate that biochemical, biophysical and morphological events occurring early in culture lead to the dedifferentiation of totipotent cells towards *de novo* organ formation of shoots and roots. The data, however, do not indicate the mechanisms for cell differentiation, neither explains why just a few cells became committed to the dedifferentiation process. Also it is unknown why in the process of bud induction and development, most of the adventitious buds stayed dormant and did not develop further, and why multiplication was not achieved, since the shoots presented an average of 2-3 axillary buds when they reached 3 cm high. More studies are needed to bring additional insights in the process of dedifferentiation of *A. amabilis*. In order to improve our present knowledge concerning these points and others, I would suggest the following:

I. Using histochemistry, biochemistry and molecular biology as a tool, studies must be undertaken using spontaneous roots to gain further insight into the rooting phenomenon. This system, if understood, has the potential to improve mass propagation through organogenesis, eliminating the painstaking multistage process since the induction of shoots/roots occurs almost simultaneously at early stages of induction and elongation of buds.

II. Also employing the same technical tools mentioned above, the application of organogenesis could be improved if further knowledge could be brought by investigating why just few totipotent parenchymatic cells located close to the medium become committed to the dedifferentiation process.

III. A detailed study of the role of combinations of cytokinins in the subsequent stage of meristemoid formation would provide valuable information for developing an improved micropropagation protocol for this species.

IV. For enhancement of multiplication it would be interesting to test bigger shoots (> 5 cm) since by that time the shoots could show better potential for axillary bud breaking out of the dormancy imposed by their apices, and then generating additional shoots in the multistage process of organogenesis. Also studies performed on the dormancy of adventitious buds could prove valuable for this goal of mass propagation.

V. Rooting is the major bottle-neck in plantlet regeneration in this species. Therefore, there is a need to study rooting process in greater detail to increase rooting to at least 50%.

VI. Since the attempt to generate somatic embryos employing mature embryos failed, it would be interesting to try different stages of immature embryos, while they are still developing inside the cone, like other studies, which have been already successfully performed with this most juvenile explant (Chalupa, 1985; Hakman *et al.*, 1985; Lu and Thorpe, 1987).

Finally, I present some additional questions that could instigate some curious mind to investigate organogenesis and embryogenesis in this species:

VII. What are the initial mechanisms that spark the *de novo* organ process? How is it that a single cell inserted in a row of similar parenchymatic cells and committed to anticlinal divisions, suddenly receives a different signal and starts to divide periclinally, against all known patterns and generates a complete new individual plant?

VIII. Why does this recalcitrant species of fir respond to the *de novo* process after a two stage pattern following subculture onto nutrient media containing combinations of cytokinins? Does this reflect a special evolutionary feature in this species? Do these combinations of hormones speed up mitotic divisions or cell cycle?

IX. What are the single or combined mechanism that induce adventitous roots in the elongating shoots while they are still attached to its original explant?

LITERATURE CITED

Abdullah, A., Yeoman, M.M., and Grace, J. (1987). Regeneration of Calabrian pine from juvenile needles. Plant. Sci. 53: 147-155.

Aitken, J. Horgan, K.J., and Thorpe, T.A. (1981). Influence of explant selection on shoot forming capacity of juvenile tissue of *Pinus radiata*. Can. J. For. Res. 11: 112-117.

Aitken-Christie, J., and Jones, C. (1987). Towards automation: radiata pine shoot hedges *in vitro*. Plant Cell Tissue Organ Cult. 8: 185-196.

Aitken-Christie, J. and Thorpe, T.A. (1984). Clonal propagation: gymnosperms. *In*: Cell Culture and Somatic Cell Genetics of Plants. Vol.I (Ed. I.K. Vasil). Academic Press, New York. pp. 82-95.

Aitken-Christie, J., Singh, A.P., Horgan, K.J., and Thorpe, T.A. (1985). Explant developmental state and shoot formation in *Pinus radiata* cotyledons. Bot. Gaz. (Chicago), 146: 196-203.

Allen, G.S., and Bientjes, W. (1954). Studies on coniferous tree seed at the University of British Columbia. For. Chron. 30: 183-196.

Ammirato, P.V. (1983). Embryogenesis. *In:* Handbook of Plant Cell Culture. Vol.I (Ed. D.A. Evans, W.R. Sharp. P.V. Ammirato and Y. Yamada). MacMillan Publishing Co., New York. pp. 82-123.

Anonymous (1978). Rules for testing seeds. J. Seed technol. 3: 1-126.

Anonymous (1985). International rules for seed testing. Seed Sci. technol. 13: 300-520.

Becwar, M.R., Wann, S.R., Johnson, M.A., Verhagen, S.A., Feirer, R.P., and Nagamani, R. (1988). Development and characterization of *in vitro* embryogenic systems in conifers. *In:* Somatic Cell Genetics of Woody Plants.(Ed. M.R. Ahuja). Kluwer Academic Publishers, Dordrecht, Netherlands.pp 1-18.

Becwar, M.R., Blush, T.D., Brown, D.W., and Chesick, E.E. (1991). Multiple paternal genotypes in embryogenic tissue derived from individual immature loblolly pine seeds. Plant Cell Tissue Organ Cult. 26: 37-44.

Bender, L., Harry, I.S., Yeung, E.C., and Thorpe, T.A. (1987). Root histology, and nutrient uptake and translocation in tissue culture plantlets and seedlings of *Thuja occidentalis* L. Trees 1: 232-237.

Berlyn, G.P., and Beck, R.C. (1980). Tissue culture as a technique for studying meristematic activity. *In:* Control of Shoot Growth in Trees. IUFRO Proceedings, July 1980, Fredericton, N.B. (Ed. C.H.A. Little). Maritimes Forest Research Centre, Fredericton, N.B. pp. 305-324.

Biondi, S., and Thorpe, T.A. (1982). Clonal propagation of forest tree species. *In:* Proceedings of the COSTED Symposium on Tissue Culture of Economically Important Plants, April 1981, Singapore. (Ed. A.N. Rao). COSTED and Asian Network for Biological Sciences, Singapore. pp. 197-204.

Blakesley, D., and Constantine, D. (1992). Uptake and metabolism of 6-benzyladenine in shoot cultures of a range of species. Plant Cell Tissue Organ Cult. 28: 183-186.

Bonga, J.M. (1977). Organogenesis in *in vitro* cultures of embryonic shoots of *Abies balsamea* (balsam fir). In Vitro 13: 41-48.

Bonga, J.M., and Durzan, D.J. (1987). Clonal propagation of mature trees: problems and possible solutions. *In:* Cell and Tissue Culture in Forestry. Vol. I. (Ed. J.M. Bonga and D.J. Durzan). Martinus Nijhoff Publishers, Boston. pp. 249-271.

Bonga, J.M., and Pond, S.E. (1991). Adventitious shoot formation in cultures of 30-year-old *Larix decidua*, *L. leptolepis*, *L. eurolepis*, and *L. laricina* trees. Plant Cell Tissue Organ Cult. 26: 45-51.

Bonner, C.A., Rodrigues, A.M., Miller, J.A., and Jensen, R.A. (1992). Physiol. Plant. 84: 319-328.

Bornman, C.H. (1983). Possibilities and constraints in the regeneration of trees from cotyledonary needles of *Picea abies* in vitro. Physiol. Plant. 57: 5-16.

Brock, T.G. and Kaufman, P.B. (1991). Growth Regulators: An account of hormones and growth regulation. *In:* Plant Physiology, A Treatise. (Ed. F.C. Steward). Vol. X: Growth and Development. (ed. R.G.S. Bidwell. Academic Press, Inc. New York, pp. 277-338.

Browder, J.O. (1992). The Limits of Extractivism. BioScience 42: 174-182.

Brown, D.C.W. and Thorpe, T.A., (1986). Plant regeneration by organogenesis. *In:* Cell Culture and Somatic Cell Genetics in Plants. Vol. 3 (Ed. I.K. Vasil). Academic Press, New York. pp. 49 - 65.

Burdett, A.N. (1990). Physiological processes in plantation establishment and the development of specifications for forest planting stock. Can. J. For. Res. 20:415-427.

Burr, K.E. (1990). The target seedling concepts: Bud dormancy and cold hardiness. *In:* Target Seedling Symposium. (Eds. R.Rose, S.J. Campbell and T.D. Landis. Proc. Combined Meeting West. For. Nurs. Assoc., USDA For. Serv. Gen. Tech. Rep. RM-200, pp.79-90.

Burr, K.E., Tinus, R.W., Wallner,S.J., and King, R.M. (1989). Relationships among cold hardiness, root growth potential and bud dormancy in three conifers. Tree Physiol. 5:291-306.

Campbell, R.A., and Durzan,D.J. (1975). Induction of multiple buds and needles in tissue cultures of *Picea glauca* by tissue culture. Can. J. Bot. 53: 1652-1657.

Cannell, M.G.R., Tabbush, P.M., Deans, J.D., Hollingsworth, M.K., Sheppard, L.J., Phillipson,J.J., and Murray, M.B. (1990). Sitka spruce and Douglas-fir seedlings in the nursery and in cold storage: root growth potential, carbohydrate content, dormancy, frost hardiness and mitotic index. Forestry 63:9-27.

Chalupa, V. and Durzan, D.J. (1973). Growth and development of resting buds of conifers *in vitro*. Can. J. For. Res. 3: 196-208.

Chalupa, V., Durzan, D.J., and Vithayasai, C. (1976). Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 2. The quantitative analysis of the growth of callus from hypocotyls and radicles. Can. J. Bot. 54: 446-455.

Chandler, S.F. and Thorpe. T.A. (1986). Hormonal Regulation of Organogenesis *In Vitro*. In: Hormonal Regulation of Plant Growth and Development. Vol. III (Ed. S.S. Purohit). Agro Botanical Publishsers (India). pp.1-27.

Chang, S., Sen, S., McKinley, C.R., Aimers-Halliday, J., and Newton, R.J. (1991). Clonal propagation of Virginia Pine (*Pinus virginiana* Mill.) by organogenesis. Plant Cell Reports 10: 131-134.

Cheah, K.T., and Cheng, T.Y. (1978). Histological analysis of adventitious bud formation in cultured Douglas-fir cotyledons. Am. J. Bot. 65: 845-849.

Cheng, T.Y. (1975). Adventitious bud formation in culture of Douglas fir (Pseudotsuga menziesii) (Mirb.) Franco). Plant Sci. lett. 5: 97-102.

Cheng, T.Y., and Voqui, T.H. (1977). Regeneration of Douglas fir plantlets through tissue culture. Science (Washington, D.C.), 198: 306-307.

Chesick, E.E., Mohn,C.A., and Hackett, W.P. (1991). Plantlet multiplication from white pine (*Pinus strobus L.*) embryos *in vitro:* bud induction and rooting. Plant Cell Tissue Organ Cult. 26: 107-114.

Christianson, M.L., and Warnick, D.A. (1983). Competence and determination in the process of *in vitro* shoot organogenesis. Dev. Biol. 95: 283-293.

Côme, D., and Thévenot, C. (1982). Environmental control of embryo dormancy and germination. *In:* The Physiology and Biochemistry of Seed Development, Dormancy and Germination (Ed. A.A. Khan). Elsevier Biomedical Press, Amsterdam, Netherlands. pp. 271-298.

Dale, J.E. (1992). How do leaves grow? BioScience 42: 423-431.

Dallimore, W., and Jackson, A.B. (1961). A handbook of Coniferae. Edward Arnold (Publ.) Ltd. London.

Danielson, H. R., and Tanaka,Y. (1978). Drying and sorting stratified ponderosa pine and Douglas-fir seeds. For. Sci. 24: 11-16.

David, A. (1982). *In vitro* propagation of gymnosperms. *In:* Tissue Culture in Forestry. (Ed. Bonga, J.M. and Durzan, D.J.). Martinus Nijhoff, London. pp. 72-108.

Debergh, P.C. (1983). Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant. 59: 270-276.

Debergh, P., Harbaqui, Y., and Lemeur, R. (1981). Mass propagation of globe artichoke (*Cynara scollymus*): evaluation of different hypotheses to overcome vitrification with special reference to water potential. Physiol. Plant. 53: 181-187.

Debergh, P.C., and Read, P.E. (1991). Micropropagation. *In:* Micropropagation, Technology and Application. (Ed. P.C. Debergh and R.H. Zimmerman) Kluwer Acad. Publ. Doedrecht, pp. 1-13.

Dhawan, V. and Bhojwani, S.S. (1987). Hardening *in vitro* and morphphysiological changes in the leaves during acclimatization of micropropagated plants of *Leucaena leucocephala* (Lam.) de Wit. Plant Sci. 53: 65-72.

Donnelly, D.J., Vidaver, W.E. and Colbow, K. (1984). Fixation of CO_2 in tissue cultured red raspberry prior to and after transfer to soil. Plant Cell Tissue Organ Cult. 4: 43-50.

Dougall, D.K. (1972). Nutrition and Metabolism. *In:* Growth, Nutrition and Metabolism of Cells in Culture. Vol. 2 (Ed. D.H. Rothblat and V.J. Christofalo). Academic Press, New York, pp. 371-406.

Dunstan, D.I. (1988). Prospects and progress in conifer biotechnology. Can. J. For. Res. 18: 1497-1506.

Dunstan, D.I., Bethune T., and Kikcio, S. (1989). *In vitro* shoot formation among 17-year-old Douglas fir provenances. Can. J. For. Res. 19: 1299-1302.

Dunstan, D.I., Lashta, D.P., Kikcio, S.I., and Thorpe, T.A. (1992). Factors affecting recurrent shoot multiplication in *in vitro* cultures of 17- to 20-year-old Douglas fir trees. In Vitro Cell. Dev. Biol. 28P: 33-38.

Dunstan, D. I., Mohammed, G.H., and Thorpe, T.A. (1986). Shoot production and elongation on explants from vegetative buds excised from 17 to 20-year-old *Pseudotsuga menziesii*. N. Z. J. For. Sci. 16: 269-282.

Dunstan, D.I. and Thorpe, T.A. (1986). Regeneration in forest trees. In: Cell Culture and Somatic Cell Genetics of Plants. (Ed. I.K. Vasil). Vol. 3. Academic Press, New York, pp. 223-241.

Durzan, D.J., and Chalupa, V. (1976a). Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 3. Growth of cells in liquid suspension cultures in light and darkness. Can. J. Bot. 54: 456-467.

______ (1976b). Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 4. Changes in amino acids in callus and in seedlings of similar genetic origin. Can. J. Bot. 54: 468-482.

______ (1976c). Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 5. Changes in free arginine and sakaguchi-reactive compounds during callus growth and in germinating seedlings of similar genetic origin. Can. J. Bot. 54: 483-495.

______ (1976d). Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 6. Free nitrogenous compounds in cell suspension cultures of jack pine as affected by light and darkness. Can. J. Bot. 54: 496-506.

Durzan, D.J., and Gupta, P.K. (1987). Somatic embryogenesis and polyembryogenesis in Douglas-fir suspension cultures. Plant Sci. 52: 229-235.

______ (1988). Somatic embryogenesis and polyembryogenesis in conifers. *In:* Biotechnology in Agriculture. (ed. A. Mizrahi) Alan R. Liss, Inc., New York, pp. 53-81.

Durzan, D. J., and Steward, F.C. (1968). Cell and tissue culture of white spruce and jack pine. Bi-mon. Res. Notes Can. For. Serv. 24: 30.

Edwards, D.G.W. (1980). Storage of prechilled *Abies* seeds. *In:* Proceedings of the IUFRO International Symposium on Forest Tree Seed Storage, September 23-27, 1980, Petawawa National Forestry Institute, Chalk River, Ont. (Ed. B.S.P. Wang and J.A. Pitel). Canadian Forestry Service, Ottawa, Ont. pp. 195-203.

______ (1981). Improving seed germination in *Abies*. Proc. Int. Plant Propag. Soc. 31: 69-78.

Ekberg, I., and Eriksson, G. (1985). Constraints to conifer breeding. *In:* Crop Physiology of Forest Trees (Ed. P.M. Tigerstedt, P. Tuttonen and V. Koski). University of Helsinki, Department of Plant Breeding. pp. 129-144.

Ellis, D.D., Barczynska, H., McCown, B.H., and Nelson, N. (1991). A comparison of BA, zeatin and thidiazuron for adventitious bud formation from *Picea glauca* embryos and epicotyl explants. Plant Cell Tissue Organ Cult. 27: 281-287.

Esau, K. (1965). Plant Anatomy (2nd ed.) John Wiley and Sons, New York.

Evans, D.A., Sharp, W.R., and Flick, C.E. (1981). Growth and behaviour of cell cultures: organogenesis and embryogenesis. *In:* Plant Tissue Culture: Methods and Applications in Agriculture (Ed. T.A. Thorpe), Academic Press, N.Y. pp. 45-113.

Fay, M.F. (1992). Conservation of rare and endangered plants using *in vitro* methods. In Vitro Cell. Dev. Biol. 28P: 1-4.

Feder, N. and O'Brien, T.P. (1968). Plant microtechnique: some principles and new methods. Am. J. Bot. 55: 123-142.

Fienberg, S.E. (1983). The analysis of cross-classified categorical data. The MIT Press, Cambridge, MA. pp. 56-94.

Flinn, B.S., Webb, D.T., and Newcomb, W. (1988). The role of cell clusters and promeristemoids in determination and competence for caulogenesis by *Pinus strobus* cotyledons *in vitro*. Can. J. Bot. 66: 1556-1565.

Franklin, J.F. (1974). Abies Mill. Fir. *In*: Seeds of Woody Plants in The United States. U.S. Department of Agriculture, Forest Service. Agriculture Handbook n°. 450.

Fridborg, G., Pederson, M., Candstrom, L., and Eriksson, T. (1978). The effect of activated charcoal on tissue cultures: adsorption of metabolites inhibiting morphogenesis. Physiol. Plant. 43: 104-106.

Gifford, D.J., Dale, P.L., and Wenzel, K.A. (1991). Lodgepole pine seed germination.III. Patterns of protein and nucleic acid synthesis in the megagametophyte and embryo. Can. J. Bot. 69: 301-305.

Gladfelter, H.J., and Phillips, G.C. (1987). *De novo* shoot organogenesis of *Pinus eldarica* Medw. *in vitro* I. Reproducible regeneration from long-term callus cultures. Plant Cell Reports 6: 163-166.

Glass, A.D.M. (1988). Nitrogen Uptake by Plant Roots. ISI Atlas Sci. Anim. Plant Sci. pp. 151-156.

Goldberg, R.B., Barker, S.J., and Perez-Grau, L. (1989). Regulation of Gene Expression during Plant Embryogenesis. Cell, 56: 149-160.

Goldfarb, B., Howe, G.T., Bailey, L.M., Strauss, S.H., and Zaerr, J.B. (1991). A liquid cytokinin pulse induces adventitious shoot formation from Douglas-fir cotyledons. Plant Cell Reports 10:156-160.

Gronroos, R. and von Arnold, S. (1985). Initiation and development of wound tissue and roots on hypocotyl cuttings of *Pinus sylvestris in vitro*. Physiol. Plant. 64: 393-401.

Gupta, P.K., and Durzan, D.J. (1985). Shoot multiplication from mature trees of Douglas fir (*Pseudotsuga menziesii*). Plant Cell Rep. 4, 177-179.

Gupta, P.K., Timmis, R., Pullman, G., Yancey, M. Kreitinger, M., Carlson, W., and Carpenter, C. (1991). Development of an embryogenic system for automated propagation of forest trees. *In* Cell Culture and Somatic Cell Genetics of Plants. (Ed. I.K. Vasil) Vol. 8 . Academic Press Inc. pp.75-93.

Hagen, S.R., Muneta, P., Augustin, J., and LeTourneau, D. (1991). Stability and utilization of picloram, vitamins, and sucrose in a tissue culture medium. Plant Cell Tissue Organ Cult. 25: 45-51.

Hakman, I., and von Arnold, S. (1985). Plantlet regeneration through somatic embryogenesis in *Picea abies* [Norway spruce]. J. Plant. Physiol. 121: 149-158.

Hakman, I.,and Fowke, L.C. (1987). Somatic embryogenesis in *Picea glauca* (white spruce) and *Picea mariana* (black spruce). Can. J. Bot. 65: 656-659.

Haissig, B.E., Davis, T.D., and Riemenschneider, D.E. (1992). Researching the controls of adventitious rooting. Physiol. Plant. 84: 310-317.

Harry, I.S., Thompson, M.R., Lu, C.Y. and Thorpe, T.A. (1987). In vitro plantlet formation from embryonic explants of eastern white cedar (*Thuja occidentalis* L.). Tree Physiol. 3: 273-283.

Harry, I.S., Thompson, M.R., and Thorpe, T.A. (1991). Regeneration of plantlets from mature embryos of western larch. In Vitro Cell. Dev. Biol. 27P:89-98.

Heaman, J.C. and Owens, J.N. (1972). Callus formation and root initiation in stem cuttings of Douglas fir (*Pseudotsuga menziesii*) Mirb. Franco. Can. J. For.t Res. 2: 121-134.

Heit, C.E. (1961). Laboratory germination and recommended testing methods for 16 spruce (*Picea*) species. Proc. Assoc. Off. Seed Anal. 51: 165-171.

Hellum, A.K. (1968). A case against cold stratification of white spruce seed prior to nursery seeding. Publ. Can. For. Serv. No. 1243.

Ishikawa, H. (1974). *In vitro* formation of adventitious buds and roots on the hypocotyl of *Cryptomeria japonica*. Bot. Mag. 87: 73-77.

Jang, J.C., and Tainter, F.H. (1991). Micropropagation of shortleaf, Virginia and loblolly x shortleaf pine hybrids via organogenesis. Plant Cell Tissue Organ Cult. 25: 61-67.

Jansson, E., and Bornman, C.H. (1981). *In vitro* initiation of adventitious structures in relation to the abscision zone in needle explants of *Picea abies:* anatomical considerations. Physiol. Plant. 53: 191-197.

Jensen, W.A. (1962). Botanical histochemistry, principles and practice. W.H. Freeman and Co., San Francisco , California.

Jensen, W.A., and Fisher, D.B. (1968). Cotton embryogenesis: the entrance and discharge of the pollen tube in the embryo sac. Planta, 78: 158-183.

Joy IV, R.W., Kumar, P.P., and Thorpe, T.A. (1991). Long-term storage of somatic embryogenic white spruce tissue at ambient temperature. Plant Cell Tissue Organ Cult. 25: 53-60.

Kevers, C., Coumans, M., Coumans-Gilles, M.F., and Gaspar, T. (1984). Physiological and biochemical events leading to vitrification of plants cultured *in vitro*. Physiol. Plant. 61: 69-74.

Klee, H., and Estelle, M. (1991). Molecular Genetic approaches to Plant Hormone Biology. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:529-551.

Konar, R.M., and Singh, M.N. (1980). Induction of shoot buds from tissue cultures of *Pinus wallichiana*. Z. Pflanzenphysiol. 99: 173-177.

Krogstrup, P. (1986). Embryolike structures from cotyledons and ripe embryos of Norway spruce (*Picea abies*). Can. J. For. Res. 16:664-668.

Kummerow, J. (1969). Unpublished report - Investigation of the role of photoperiod in the rooting and growth induction of short shoots of pine. U.S. Forest Service Project No. 54-FS-3.

Kuoksa, T., and Hohtola, A. (1991). Freeze-preservation of buds from Scots pine trees. Plant Cell Tissue Organ Cult. 27: 89-93.

Kvaalen, H., and von Arnold, S. (1991). Effects of various partial pressures of oxygen and carbon dioxide on different stages of somatic embryogenesis in *Picea abies*. Plant Cell Tissue Organ Cult. 27: 49-57.

Lavender, D.P. (1990). Measuring phenology and dormancy. *In:* Techniques and Approaches in Forest Tree Ecophysiology, (Eds. J.P. Lassoie and T.M. Hinckley). CRC Press, Boca Raton, FL, pp.403-422.

Leadem, C.L. (1986). Stratification of *Abies amabilis* seeds. Can. J. For. Res. 16: 755-760.

Lelu, M.A.P. and Bornman, C.H. (1990). Induction of Somatic embryogenesis in excised cotyledons of *Picea glauca* and *Picea mariana*. J. Plant. Physiol. 28 (6): 785-791.

Lelu, M.A.P., Boulay,M., and Arnaud, Y. (1987). Obtention de cals embryogènes à partir de cotyledons de *Picea abies* [L.] Karst. prélevés sur de jeunes plantes âgées de 3 à 7 jours après germination. C.R. Acad. Sci. Ser. 3, 305:105-109.

Lin, Y., Wagner, M.R., and Heidmann, L.J. (1991). *In vitro* formation of axillary buds by immature shoots of Ponderosa pine. Plant Cell Tissue Organ Cult. 26: 161-166.

Lu, C.-Y., and Thorpe, T.A. (1987). Somatic embryogenesis and plant regeneration in cultured immature embryos of *Picea glauca*. J. Plant Physiol. 128: 297-302.

MacGaw, B.A. (1987). Cytokinin biosynthesis and metabolism. *In:*Plant Hormones and Their Role in Plant Growth and Development. (ed. P.J. Davies). Martinus Nijhoff Publishers. Dordrecht, pp. 76-93.

Maretzki, A., Thom, M., and Nickell, L.G. (1974). Utilization and metabolism of carbohydrates in cell and callus cultures.*In:* Tissue Culture and Plant Science (Ed. H.E. Street). Academic Press, London.

Margolis, H.A., and Brand, D.G. (1990). An ecophysiological basis for understanding plantation establishment. Can. J. For. Res. 20:375-390.

Menk, D.W. (1991). Perspectives on Genetic Analysis of Plant Embryogenesis. Plant Cell. 3: 857-866.

Messner, B., Boll, M., and Berndt. J. (1991). l-Phenylalanine ammonia-lyase in suspension culture cells of spruce (*Picea abies*). Induction by UV-light and fungal elicitor. Plant Cell Tissue Organ Cult. 27: 267-274.

Mohammed, G.H., and Vidaver, W.E. (1988). Root production and plantlet development in tissue-cultured conifers. Plant Cell Tissue Organ Cult. 14: 137-160.

______ (1991). Plantlet morphology and the regulation of net water loss in tissue-cultured Douglas-fir. Physiol. Plant. 83: 117-121.

Mohammed, G.H., Gillies, S.L. and Vidaver, W.E. (1992). *Ex vitro* photosynthetic activity in plantlets of tissue-cultured Douglas-fir. Tree Physiol. 10, 403-410.

Montain, C.R.; Haissig, B.E. and Curtis, J.D. (1983a). Initiation of adventitious root primordia in very young *Pinus banksiana* seedling cuttings. Can. J. For. Res. 13: 191-195.

______ (1983b). Differentiation of adventitious root primordia in callus of *Pinus banksiana* seedling cuttings. Can. J. For. Res. 13: 195-200.

Mott, R.L., Smelter, R.H., and Mehra-Palta, A. (1977). An anatomical and cytological perspective on pine organogenesis *in vitro*. Tappi, pp. 9-14.

Mott, R.L., Smelter, R.H., Mehra-Palta, A., and Zobel, B.J. (1977). Production of forest trees by tissue culture. Tappi, 60: 62-64.

Murashige, T. (1974). Plant propagation through tissue culture. Ann. Rev. Plant Physiol. 25: 135-166.

______ (1978). Impact of plant tissue culture on agriculture. *In:* Frontiers of Plant Tissue Culture (Ed. T. A. Thorpe). University of Calgary, Calgary-Canada, pp. 15-26.

______ (1990). Plant propagation by tissue culture: a practice with unrealized potential. *In:* Handbook of Plant Cell Culture, vol. 5: Ornamental species (Eds. P.V. Ammirato, D.A. Evans, W.R. Sharp and Y.P.S. Bajaj). McGraw Hill Publ. Co., N.Y. pp. 3-9.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473-479.

Oaks, A. (1992). A re-evaluation of nitrogen assimilation in roots. BioScience 42: 103-111.

O'Brien, T.P., and McCully, M.E. (1981). The Study of Plant Structure: Principles and Selected Methods. Termarcarphi Pty. Ltd., Melbourne, Australia.

Ohira, K., Ikeda, M., and Ojima, K. (1976). Thiamine requirements of various plant cells, in suspension culture. Plant Cell Physiol. 17: 583.

Owen, J.N., and Miller, A.R. (1992). An examination and correction of plant tissue culture basal medium formulations. Plant Cell Tissue Organ Cult. 28: 147-150.

Owens, H.R., and Molder, M. (1984a). The reproductive cycle of interior spruce. Information Services Branch, B.C. Ministry of Forests, Victoria.

______ (1984b). The reproductive cycle of lodgepole pine. Information Services Branch, B.C. Ministry of Forests, Victoria.

Pappas, P.W. (1971). The use of a chrome alum-gelatin (subbing) solution as a general adhesive for paraffin sections. Stain Technology 46, 121-124.

Patel, K.R. and Berlyn, G.P. (1983). Cytochemical investigations on multiple bud formation in tissue cultures of *Pinus coultieri*. Can. J. Bot. 61: 575-585.

Patel, K.R., and Thorpe, T.A. (1984a). *In vitro* differentiation of plantlets from embryonic explants of lodgepole pine (*Pinus contorta* Dougl. ex Loud). Plant Cell Tissue Organ Cult. 3: 131-142.

______ (1984b). Histochemical examination of shoot initiation in cultured cotyledon explants of radiata pine. Bot. Gaz. (Chicago), 145: 312-322.

______ (1986). *In vitro* regeneration of plantlets from embryonic and seedling explants of Engelmann spruce (*Picea engelmannii* Parry). Tree Physiol. 1: 289-301.

Patel, K.R., Kim, H.R., and Thorpe, T.A. (1986). Plantlet formation in pitch pine (*Pinus rigida* Mill.) by tissue culture methods. For. Ecol. Manage. 15: 147-160.

Pérez-Bermudez, P., and Sommer, H.E. (1987). Factors affecting adventitious bud induction in *Pinus elliottii* (Engelm.) embryos cultured *in vitro*. Plant Cell Tissue Organ Cult. 11: 25-35.

Pierik, R.L.M. (1987). *In Vitro* Culture of Higher Plants. Martinus Nijhoff Publishers, Dordrecht. 344 pp.

Potrykus, I. (1991). Gene transfer to plants: Assessment of published approaches and results. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:205-225.

Pulido, C.M., Harry, I.S., and Thorpe, T.A. (1990). *In vitro* regeneration of plantlets of Canary Island pine (*Pinus canariensis*). Can. J. For. Res. 20: 1200-1211.

Quoirin, M., and LePoivre, P. (1977). Études de milieux adaptés aux cultures *in vitro* de *Prunus*. Acta Hortic. 78: 437-442.

Redenbaugh, K., Fujii, J.A., and Slade, D. (1991). Synthetic Seed Technology. *In:* Cell Culture and Somatic Cell Genetics, vol. 8, (ed. I.K. Vasil) Academic Press Inc., pp. 35-74.

Reilly, K.J., and Washer, J. (1977). Vegetative propagation of radiata pine by tissue culture: plantlet formation from embryonic tissue. N.Z. J. For. Sci. 7: 199-206.

Reines, M. and McAlpine, R.G. (1959). The morphology of normal callused and rooted shoots of slash pine. Bot. Gaz. 121: 118-124.

Ritchie, G.A. (1989). Integrated growing schedules for achieving physiological uniformity in coniferous planting stock. Forestry Suppl. 62:213-227.

Ritchie, G.A., Tanaka,Y., and Duke, S.D. (1992). Physiology and morphology of Douglas-fir rooted cuttings compared to seedlings and transplants. Tree Physiol. 10:179-194.

Rumary, C., Patel, K.R., and Thorpe, T.A. (1985). Plantlet formation in black and white spruce. II. Histological analysis of adventitious shoot formation *in vitro*. Can. J. Bot. 64: 997-1002.

Rumary, C., and Thorpe, T.A. (1984). Plantlet formation in black and white spruce. I. *In vitro* techniques. Can. J. For. Res. 14: 10-16.

Saravitz, C.H. (1990). *In vitro* propagation of Fraser fir (*Abies fraseri*) and Virginia pine (*Pinus virginiana*) from embryonic tissues. Ph.D. thesis, North Carolina State University, Raleigh.

Saravitz, C.H., Blazich, F.A., and Amerson, H.V. (1987). *In vitro* propagation of Fraser fir (*Abies fraseri*). HortScience, 22: 1068 (Abstr.).

______ (1991). *In vitro* propagation of Fraser fir from embryonic explants. Can. J. For. Res. 21: 404-409.

Schenk, R.V., and Hildebrandt, A.C. (1972). Medium and techniques for induction and growth of monocotyledoneous and dicotyledonous plant cell cultures. Can. J. Bot. 50:199-204.

Schenk, N., Hsiao,K.C., and Bornman,C.H. (1991). Avoidance of precipitation and carbohydrate breakdown in autoclaved plant tissue culture media. Plant Cell Reports 10:115-119.

Schuch, W. (1991). Advances in plant biotechnology and their implication for forestry research. In Vitro Cell Dev. Biol. 27P: 99-103.

Schuller, A., Reuther, G., and Geier, T. (1989). Somatic embryogenesis from seed explants of *Abies alba*. Plant Cell Tissue Organ Cult. 17: 53-58.

Simola, L.K., Lemmetynen,J., and Santanen, A. (1992). Lignin release and photomixotropism in suspension cultures of *Picea abies*. Physiol. Plant. 84: 374-379.

Smith, D.R. (1975). Root initiation in *Pinus radiata*. Ph.D Thesis, Univ. Calgary. pp. 240.

Smith, D.R. and Thorpe, T.A. (1975). Root initiation in cuttings of Pinus radiata seedlings. I. Developmental sequence. J. Exp. Bot. 26: 184-192.

Sommer, H.E., Brown,C.L. and Caldas, L.S. (1981). *In vitro* methods applied to forest trees. *In:* Plant Tissue Culture: methods and applications in agriculture. (Ed. T.A. Thorpe). Academic Press, New York. pp. 349-358.

Sommer, H.E., Brown, C.L., and P.P. Kormanik (1975). Differentiation of plantlets in longleaf pine (*Pinus palustris* Mill) tissue cultured *in vitro*. Bot. Gaz. (Chicago), 136: 196-200.

Sorensen, F.C. (1980). Effect of date of cone collection and stratification period on germination and growth of Douglas-fir seeds and seedlings. USDA For. Serv. Res. Note PNW-346.

Tautorus, T.E., Fowke, L.C., and Dunstan, D.I. (1991). Somatic embryogenesis in conifers. Can. J. Bot. 69: 1873-1899.

Teasdale, R.D., and Richards, D.K. (1991). Study of a factor produced by suspension-cultured *Pinus radiata* cells which enhances cell growth at low inoculum densities. Low inoculum growth factor from *Pinus radiata*. Plant Cell Tissue Organ Cult. 26: 53-59.

Timmis, R. (1985). Factors influencing the use of clonal material in commercial forestry. *In:* Crop Physiology of Forest Trees (Ed. P.M. Tigerstedt, P. Tuttonen and V. Koski). University of Helsinki, Department of Plant Breeding. pp. 259-272.

Thorpe, T.A. (1980). Organogenesis *in vitro*: Structural, physiological and biochemical aspects. In: Perspectives in Plant Cell and Tissue Culture. (Ed. Vasil, I.K.). Int. Rev. Cytol., Supplement 11A. pp. 87-101.

______ (1983a). Biotechnological applications of tissue culture to forest tree improvement. *Biotechnol. Adv.* 1, 263- 278.

______ (1983b). Morphogenesis and Regeneration in Tissue Culture. *In:* Genetic Engineering: Applications to Agriculture (Ed. L.D. Owens). Rowman & Allanheld, Totowa pp. 285-303.

______ (1985). Clonal propagation of conifers. Proc. Int. Symp. *In Vitro* Propag. Forest Tree Species, pp. 33-50. Azienda Regionale delle Foreste, Bologna, Italy.

______ (1988). *In vitro* somatic embryogenesis. ISI Atlas Sci. Anim. Plant Sci. 1: 81-88.

______ (1990). Organogenesis: Structural, physiological and biochemical aspects. *In:* Plant Aging: Basic and Applied Approaches.(Eds. R. Rodrigues, R.T. Sanches and D.J. Durzan). Nato ASI Series A: Life Sciences Vol. 186. Plenum Press, New York, pp. 191-197.

Thorpe, T.A. and Biondi, S. (1981). Regulation of plant organogenesis. In: Advances in Cell Culture. Vol.I. (Ed. A.N. Rao). Academic Press, Inc. pp. 213-239.

Thorpe, T.A. and Biondi, S. (1984). Conifers. *In:* Handbook of Plant Cell Culture. Vol.2. Crop species. (Ed. W.R. Sharp, D.A. Evans, P.V. Ammirato and Y. Yamada). MacMillan, New York. pp.435-470.

Thorpe, T.A. and Hasnain, S. (1988). Micropropagation of conifers: methods, opportunities and costs. *In*: Tree Improvement- Progressing Together. Proceedings of the 21st Meeting of the Canadian Tree Improvement Association, 1987, Truro, N.S. (Ed. E.K. Morgenstern and J.B. Boyle). Canadian Forestry Service, Chalk River, pp. 68-84.

Thorpe,T.A., and Harry (1990). Special problems and prospects in the propagation of woody species. *In:* Plant Aging: Basic and Applied Approaches.(Eds. R. Rodrigues, R.T. Sanches and D.J. Durzan). Nato ASI Series A: Life Sciences Vol. 186. Plenum Press, New York, pp. 67-74.

Thorpe, T.A., Harry, I.S. and Kumar, P.P. (1991). Application of micropropagation to forestry. *In:* Micropropagation, Technology and Application. (Eds. P.C. Debergh and R.H. Zimmerman). Kluwar Academic Publishers, Dordrecht, pp. 311-336.

Thorpe, T.A., and Patel,K.R. (1984). Clonal propagation: adventitious buds. *In:* Cell Culture and Somatic Cell Genetics of Plants, vol.I, Laboratory Procedures and Their Applications. (Ed.I.K.Vasil) Academic Press, New York. pp.49-58.

______ (1986). Comparative morpho-histological studies on the sites of shoot initiation in various conifer explants. New Zealand Journal of Forestry Science 16: 257-268.

Toivonen, P.M.A., and Kartha, K.K. (1988). Regeneration of plantlets from *in vitro* cultured cotyledons of white spruce (*Picea glauca* (Moench) Voss). Plant Cell Rep. 7: 318-321.

Torrey, J.G. (1966). The initiation of organized development in plants. Adv. in Morphogenesis 5: 39-91.

Tremblay, L. (1990). Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. Can. J. Bot. 68: 236-242.

Tremblay, L., and Tremblay, F.M. (1991). Carbohydrate requirements for the development of black spruce (*Picea mariana* (Mill.) B.S.P.) and red spruce (*P. rubens* Sarg.) somatic embryos. Plant Cell Tissue Organ Cult. 27: 95-103.

Tung, C.-H., Wisniewski, L., and DeYoe, D.R. (1986). Effects of prolonged cold storage on phenology and performance of Douglas-fir and noble fir 2+0 seedlings from high-elevation sources. Can. J. For. Res. 16:471-475.

Vapaavuori, E.M., Rikala, R. and Ryyppö, A. (1992). Effects of root temperature on growth and photosynthesis in conifer seedlings during shoot elongation. Tree Physiol. 10, 217-230.

Vasil, I.K. and Vasil, V. (1980). Clonal propagation. Intl. Rev. Cytl. Suppl. 11A:145-173.

Villalobos, V.M.A. (1983). Organogenesis in Radiata Pine Cotyledons. Ph.D Thesis, Univ. Calgary. pp. 157.

Villalobos, V.M., Oliver, M.J., Yeung, E.C., and Thorpe, T.A. (1984). Cytokinin-induced switch in development in excised cotyledons of radiata pine cultured *in vitro*. Physiol. Plant. 61: 483-489.

Villalobos, V.M., Yeung, E.C., and Thorpe, T.A. (1985). Origin of adventitious shoots in excised radiata pine cotyledons cultured *in vitro*. Can. J. Bot. 63: 2172-2176.

von Arnold, S. (1982). Vegetative propagation of *Picea abies*. *In:* Procedings of the 5th International Congress of Tissue and Cell Culture, 1982, Tokyo.(Ed. A. Fujiwara). Maruzen Co. Ltd., Tokyo, pp. 753-754.

von Arnold, S. and Eriksson, T. (1978). Induction of adventitious buds on embryos of Norway spruce grown *in vitro*. Physiol. Plant. 44: 283-287.

______ (1981). *In vitro* studies of adventitious shoot formation in *Pinus contorta*. Can. J. Bot. 59: 870-874.

______ (1984). Effect of agar concentration on growth and anatomy of adventitious shoots of *Picea abies* (L.) Karst. Plant Cell Tissue Organ Cult. 3: 257-264.

Wagley, L.M., Gladfelter, H.J., and Phillips, G.C. (1987). *De novo* shoot organogenesis of *Pinus eldarica* Medw. *in vitro* II. Macro- and micro-photographic evidence of *de novo* regeneration. Plant Cell Reports 6: 167-171.

Wang, B.S.P. (1976). Dormancy and laboratory germination criteria of white spruce seed. *In:* Proceedings of the 2nd. International Symposium on Physiology of Seed Germination, December 1976. Tokyo, Japan. Government Forest Experiment Station, Ministry of Agriculture and Forestry, Tokyo, Japan. pp. 179-186.

Warmke, H.E., and Lee, J.S. (1976). Improved staining procedures from semithin epoxy sections of plant tissues. Stain Technology 51: 179-185.

Webb, D.T., and Diaz Santiago, O. (1983). Cytokinin-induced bud formation on Caribbean pine (*Pinus caribeae*) embryos *in vitro*. Plant Sci. Lett. 32: 17-21

Wolswinkel. P. (1988). Nutrient Transport into Developing Seeds. ISI Atlas Sci. Anim. Plant Sci. 1: 298-302.

Yeung, E.C. (1984). Histological and Histochemical Staining Procedures. *In*: Cell Culture and Somatic Cell Genetics of Plants. Vol. 1. Laboratory Procedures and their Applications. (Ed. I.K. Vasil). American Press, Orlando, Florida.

Yeung, E.C., Aitken, J., Biondi, S. and Thorpe, T.A. (1981). Shoot histogenesis in cotyledon explants of radiata pine. Bot. Gaz. (Chicago) 142: 494-501.

Yeung, E.C., and Law, S.K. (1987). Serial sectioning techniques for a modified LKB historesin. Stain Technology Vol. 62, No. 3. pp. 147-153.

Zobel, B., and Talbert, J. (1984). Applied Forest Tree Improvement. John Wiley and Sons, New York.

Zar, J.H. (1984). Biostatistical Analyses. 2nd edition. Prentice-Hall, N.J.

Zygmont, N., and Schwarz, O.J. (1987). *In vitro* adventitious bud formation on cultured embryos of *Abies fraseri*. In Vitro, 23: 49A (Abstr.).

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *In vitro* PROPAGATION OF PACIFIC SILVER FIR (*Abies amabilis*) FROM EMBRYONIC EXPLANTS submitted by LUIZ KULCHETSCKI in partial fulfillment of the requirements for the degree of Doctor of Phylosophy.

Trevor A. Thorpe

T.A. Thorpe, Supervisor

F.C. Yeh, Supervisor

Victor Lieffers

V.J. Lieffers

James Beck

J.A. Beck

A. Flanagan

J. Zwiazek

David I. Dunstan

D.I. Dunstan, External Examiner
Plant Biotechnology Institute, NRC - Saskatoon

Date Nov 26'92